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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN AND WEST; Commercial sequence data bases for SEQ ID NOS : 1 & 2.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Database on Genbank, NAGASE et al. Prediction of the cosing sequences of unidentified human genes. XV. The complete sequences of 100 new cDNA clones from brain which codes for large proteins in vitro. 11 November 1999, Accession No. AB033025. [Accession No. AB033025 is 74.2% identical to Applicants' SEQ ID NO : 1 and encodes a protein Q9UlM1 which is 74.4% identical to Applicants' SEQ ID NO : 2].	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier application or patent published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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GENE ASSOCIATED WITH BONE DISORDERS

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Applications 60/309,495 (filed August 3, 2001) and 60/317,975 (filed September 10, 2001); all of which are herein incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

Living bone tissue is continuously being replenished by the processes of resorption and deposition of bone matrix and minerals. This temporally and spatially coupled process, termed bone remodeling, is accomplished largely by two cell populations, osteoclasts and osteoblasts. The remodeling process is initiated when osteoclasts are recruited from the bone marrow or the circulation to the bone surface to remove a disk-shaped packet of bone producing an area of resorbed surface. A team of osteoblasts recruited to the resorbed bone surface from the bone marrow subsequently replaces the bone matrix and mineral. Among the pathological conditions associated with abnormal bone cell function is osteoporosis, a diseased characterized by reduced amounts of bone (osteopenia) and increased bone fragility. These changes can be the result of increased recruitment and activity of osteoclasts, in combination with reduced recruitment or activity of osteoblasts (Teitelbaum *et al.* (1997) J. Leukoc. Biol. 61, 381-388; Simonet *et al.* (1997) Cell 89, 309-319).

A very significant patient population that would benefit from new therapies designed to promote bone formation or inhibit resorption are those patients suffering from osteoporosis. Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at menopause, while osteoporosis type II is associated with advancing age. An estimated twenty to twenty-five million people are at increased risk for fracture because of site-specific bone loss. The cost of treating osteoporosis in the United States is currently estimated to be in the order of ten billion dollars per year. Demographic trends, *i.e.*, the gradually increasing age of the United States population, suggest that these costs may increase up to three fold by the year 2020 if a safe and effective treatment is not found.

Bone resorption is initiated with the destruction of bone matrix by osteoclasts. Following this initial phase of bone destruction, or resorptive phase, formation of new bone protein matrix begins. New bone proteins are deposited, and sometime later, minerals begin to be incorporated into the newly formed matrix. The formation of bone matrix and its subsequent mineralization are exclusive functions of osteoblasts.

In theory, either decreased bone formation relative to resorption or increased bone resorption relative to formation can cause the net loss of bone in osteoporosis. Control of the rate of breakdown and synthesis of new bone tissue is critical to the integrity of the skeletal structure. When the rates become unbalanced, serious conditions may result. Although there is always a net excess of bone resorption in osteoporosis, the absolute amounts of bone formation and resorption can vary from case to case.

SUMMARY OF THE INVENTION

Few treatments are available to modulate the formation and resorption processes of bone maintenance and development. In bone disorders such as osteoporosis, it may be useful to monitor or modify the expression levels or activities of genes involved in bone formation or resorption. The present inventors have examined cell populations comprising precursor stem cells and cell populations comprising precursor stem cells that have been induced to differentiate into osteoblasts and have discovered that the expression of previously unidentified gene changes during this differentiation process. This change in gene expression provides a useful marker for diagnostic and prognostic uses as well as a marker that can be used for drug screening and therapeutic indications.

The invention encompasses an isolated nucleic acid molecule selected from the group consisting of: an isolated nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1; an isolated nucleic acid molecule encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 2; an isolated nucleic acid molecule that encodes a polypeptide fragment of at least about 1,015 amino acids of SEQ ID NO: 2; and an isolated nucleic acid molecule that encodes a polypeptide that exhibits at least about 75% amino acid sequence identity to SEQ ID NO: 2 over the entire contiguous sequence. In a preferred embodiment of the invention, the isolated nucleic acid comprises nucleotides 251-4,336 of SEQ ID NO: 1.

In some embodiments, the isolated nucleic acid molecule is operably linked to one or more expression control elements. The invention also includes a vector comprising an isolated nucleic acid molecule and a host cell transformed to contain the nucleic acid molecule. The host cell may be either eukaryotic or prokaryotic.

The invention also encompasses a method for producing a polypeptide comprising culturing a host cell transformed with the aforementioned nucleic acid molecule under conditions in which the polypeptide encoded by said nucleic acid molecule is expressed and the isolated polypeptide produced by this method.

The invention further encompasses an isolated polypeptide selected from the group consisting of: an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 2; an

isolated polypeptide comprising a fragment of at least 1,015 amino acids of SEQ ID NO: 2; an isolated polypeptide comprising conservative amino acid substitutions of SEQ ID NO: 2; and an isolated polypeptide exhibiting at least about 75% amino acid sequence identity with SEQ ID NO: 2. In some embodiments, the isolated polypeptide comprises amino acids 1 to 348 of SEQ ID NO: 2. The invention includes isolated antibodies that bind to the aforementioned polypeptide. The antibodies may be either monoclonal or polyclonal.

The invention encompasses a method of screening for an agent that modulates the differentiation of a population of stem cells into osteoblast cells or is capable of increasing bone density comprising: exposing a population of stem cells to the agent, and measuring expression or activity of a polypeptide encoded by the nucleic acid of the invention following exposure to the agent, wherein an decrease in the level of expression or activity is indicative of an agent capable of stimulating stem cells to differentiate into osteoblast cells or increasing bone density.

In yet another embodiment, the invention includes a method of diagnosing a condition characterized by abnormal stem cell differentiation or bone density comprising detecting in a stem cell sample the level of expression or activity of a polypeptide encoded by the nucleic acid of the invention, wherein abnormal expression or activity is indicative of a condition characterized by abnormal stem cell differentiation or bone cell density. In a preferred embodiment, the condition is osteoporosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a graphical representation of the expression level of the target mRNA of SEQ ID NO: 1 in human fetal stromal cells as assayed using READS gel analysis in response to treatment with the osteogenic agent TGF-β1 (1 ng/ml) versus untreated controls. Figure 1A shows the effect over a time period of twenty-four days whereas Figure 1B shows effects over a period of forty-eight hours.

Figure 2 provides a graphical representation of the expression level of the target mRNA of SEQ ID NO: 1 in human fetal stromal cells assayed using READS gel analysis in response to treatment with the osteogenic agent BMP-2 (300 ng/ml) versus untreated controls. Figure 2A shows the effect over a time period of twenty-four days whereas Figure 2B shows effects over a period of forty-eight hours.

Figure 3 provides a graphical representation of the expression level of the target mRNA of SEQ ID NO: 1 in human mesenchymal stem cells as assayed using READS gel analysis in response to treatment with osteogenic and adipogenic agents. In Figure 3A, cells were cultured in a medium supplemented with 10% fetal calf serum with or without dexamethasone (100 nM) for time period ranging from zero to seven days. In Figure 3B, cells were cultured in the same

manner with or without BMP-2 (200 ng/ml) for the same time period. In Figure 3C, cells were cultured in a medium containing 10% rabbit serum with or without addition of dexamethasone (100 nM) for the same time period.

Figure 4 provides a graphical representation of the expression levels of the target mRNA of SEQ ID NO: 1 in human fetal stromal cells (A, B) and in human mesenchymal stromal cells (C) as assayed by quantitative RT-PCR. In Figure 4A, cells were cultured using non-mineralization conditions in the absence (control, open circles with dotted line) or presence of either 1 ng/ml TGF-β1 (closed circles) or 300 ng/ml of BMP-2 (closed triangles) for time periods up to six days. In Figure 4B, cells were cultured using mineralization conditions in the absence of the same agents as in Figure 4A for time periods up to twenty-one days (504 hours). In Figure 4C, mesenchymal stem cells were cultured in media supplemented with ascorbic acid and β-glycerophosphate in the absence and presence of either 1 ng/ml TGF-β1, 200 ng/ml BMP-2 or 100 nM dexamethasone for time periods up to sixteen days (384 hours).

Figure 5 shows expression levels, depicted as Ct values, of the target mRNA of SEQ ID NO: 1 in various human tissues as assayed using TaqMan quantitative RT-PCR methods. Expression levels of the target mRNA in resting human fetal stromal cells (HFSC control) and human mesenchymal stem cells (MSC control) is also provided.

Figure 6 displays a graphical representation of the results of a hydrophobicity analysis of the polypeptide of SEQ ID NO: 2.

Figure 7 shows a Northern blot in which the expression level of SEQ ID NO: 1 was measured in several normal human tissues including brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung and in leukocytes (ClonTech human mRNA blot-H12). RNA markers are present on the left side of the blot.

Figure 8 shows a Northern blot in which the expression level of SEQ ID NO: 1 was measured in human tissues as well as in human fetal stromal cells (FSC) and mesenchymal stem cells (MSC) treated with control or osteogenic agents.

Figure 9 provides a graphical representation of the effects of inhibition of target mRNA expression on osteoblast differentiation, as measured by alkaline phosphatase expression. In Figure 9A treatment of 100 ng/ml BMP-2 treated cells with siRNA duplex increases alkaline phosphatase expression by 414% compared to control duplex treated cells. Figure 9B demonstrates that the inhibition of target mRNA expression by the same siRNA duplex was 73% compared to control duplex.

DETAILED DESCRIPTION

General Description

The present invention is based in part on the identification of a new gene family that is differentially expressed in bone deposition disorders. This gene, designated 76032, corresponds to the human cDNA of SEQ ID NO: 1. Genes that encode the human protein of SEQ ID NO: 2 may also be found in other animal species, particularly mammalian species. This novel gene has been identified as being differentially regulated during the maturation of osteoblasts and its expression is correlated, for example, with bone deposition disorders such as osteoporosis.

Further, monitoring of expression may be used for disease diagnosis and may be indicative of treatment efficacy. The nucleic acid molecules of SEQ ID NO: 1 or its fragments, as well as the peptides they encode, can serve as targets for agents that can be used to modulate the activity of the proteins and nucleic acids of the invention, such as the protein having the amino acid sequence of SEQ ID NO: 2. For example, agents may be identified which bind to the proteins and nucleic acids of the invention and modulate biological processes associated with bone deposition such as differentiation of stem cells into osteoblasts.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, the term "bone density" refers to the mass or quantity of bone tissue in a certain volume of bone.

As used herein, the term "bone deposition" refers the formation of new bone during osteogenesis.

As used herein, the term "bone resorption" refers to a decrease in bone density and/or mass. Generally, mechanisms of bone resorption include, but are not limited to, secretion of enzymes and/or acids by osteoclasts to facilitate the breakdown of bone.

As used herein, the term "osteoporosis" refers to a pathological disorder characterized by a reduction in the amount of bone mass and/or density. Osteoporosis is generally characterized by increased osteoclast activity and/or decreased osteoblast activity.

As used herein, the term "stem cell" or "mesenchymal stem cell" refers to a cell capable of differentiation into an osteoblast cell. These terms are used interchangeably throughout the specification to indicate that the cell is undifferentiated.

As used herein, the terms "stem cell differentiation" and "osteoblast differentiation" refers to the process in which a stem cell develops specialized functions during maturation into an osteoblast cell.

As used herein, the term "osteoblast" refers to a cell capable of mediating bone deposition. Osteoblasts are derived from mesenchymal stem cells of the bone marrow stroma.

As used herein, the term "osteoclast" refers to a cell capable of mediating bone resorption.

Nucleic Acid Molecules

The present invention further provides nucleic acid molecules that encode the protein having SEQ ID NO: 2 and the related proteins herein described, preferably in isolated form. As used herein, "nucleic acid" is defined as RNA or DNA that encodes a protein or peptide as defined above, is complementary to a nucleic acid sequence encoding such peptides, hybridizes to SEQ ID NO: 1 across the open reading frame under appropriate stringency conditions, or encodes a polypeptide that shares at least about 75% sequence identity, preferably at least about 80%, more preferably at least about 85%, and even more preferably at least about 90% or even 95% or more identity with the entire contiguous amino acid sequence of SEQ ID NO: 2. The "nucleic acid" of the invention further includes nucleic acid molecules that share at least 80%, preferably at least about 85%, and more preferably at least about 90% or 95% or more identity with the nucleotide sequence of SEQ ID NO: 1, particularly across the open reading frame. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative backbones or including alternative bases whether derived from natural sources or synthesized. Such nucleic acids, however, are defined further as being novel and unobvious over any prior art nucleic acid including that which encodes, hybridizes under appropriate stringency conditions, or is complementary to nucleic acid encoding a protein according to the present invention.

Homology or identity at the nucleotide or amino acid sequence level is determined by **BLAST** (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs **blastp, blastn, blastx, tblastn** and **tblastx** (Altschul *et al.* (1997) Nucleic Acids Res. 25, 3389-3402 and Karlin *et al.* (1990) Proc. Natl. Acad. Sci. USA 87, 2264-2268, both fully incorporated by reference) which are tailored for sequence similarity searching. The approach used by the **BLAST** program is to first consider similar segments, with and without gaps, between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of

sequence databases, see Altschul *et al.* (1994) Nature Genetics 6, 119-129 which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (*i.e.*, the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter (low complexity) are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the **BLOSUM62** matrix (Henikoff *et al.* (1992) Proc. Natl. Acad. Sci. USA 89, 10915-10919, fully incorporated by reference), recommended for query sequences over 85 in length (nucleotide bases or amino acids).

For **blastn**, the scoring matrix is set by the ratios of **M** (*i.e.*, the reward score for a pair of matching residues) to **N** (*i.e.*, the penalty score for mismatching residues), wherein the default values for **M** and **N** are +5 and -4, respectively. Four **blastn** parameters were adjusted as follows: Q=10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every winkth position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent **Blastp** parameter settings were Q=9; R=2; wink=1; and gapw=32. A **Bestfit** comparison between sequences, available in the GCG package version 10.0, uses DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty) and the equivalent settings in protein comparisons are GAP=8 and LEN=2.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer (pH 6.5) with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is hybridization in 50% formamide, 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5× Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2× SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal. Preferred molecules are those that hybridize under the above conditions to the complement of SEQ ID NO: 1 and which encode a functional protein. Even more preferred hybridizing molecules are those that hybridize under the above conditions to the complement strand of the open reading frame of SEQ ID NO: 1.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid molecules encoding other polypeptides.

The present invention further provides fragments of the encoding nucleic acid molecule. As used herein, a fragment of an encoding nucleic acid molecule refers to a small portion of the

entire protein coding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the protein, the fragment will need to be large enough to encode the functional regions of the protein. For instance, fragments which encode peptides corresponding to predicted antigenic regions may be prepared. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming.

Fragments of the encoding nucleic acid molecules of the present invention (*i.e.*, synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding proteins of the invention, can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci *et al.* (1981) J. Am. Chem. Soc. 103, 3185-3191 or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the gene, followed by ligation of oligonucleotides to build the complete modified gene. In a preferred embodiment, the nucleic acid molecule of the present invention contains a contiguous open reading frame of at least about three-thousand and forty-five nucleotides.

The encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. A variety of such labels are known in the art and can readily be employed with the encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides and the like. A skilled artisan can readily employ any such label to obtain labeled variants of the nucleic acid molecules of the invention. Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence encoded by a nucleic acid falling within the contemplated scope of the present invention.

Isolation of Other Related Nucleic Acid Molecules

As described above, the identification and characterization of the nucleic acid molecule having SEQ ID NO: 1 allows a skilled artisan to isolate nucleic acid molecules that encode other members of the protein family in addition to the sequences herein described.

For instance, a skilled artisan can readily use the amino acid sequence of SEQ ID NO: 2 to generate antibody probes to screen expression libraries prepared from appropriate cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified

protein (as described below) or monoclonal antibodies can be used to probe a mammalian cDNA or genomic expression library, such as lambda gtll library, to obtain the appropriate coding sequence for other members of the protein family. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme.

Alternatively, a portion of the coding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the protein family from any mammalian organism. Oligomers containing approximately 18-20 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives.

Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively clone an encoding nucleic acid molecule. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other encoding nucleic acid molecules.

Nucleic acid molecules encoding other members of the protein family may also be identified in existing genomic or other sequence information using any available computational method, including but not limited to: PSI-BLAST (Altschul *et al.* (1997) Nucleic Acids Res. 25:3389-3402); PHI-BLAST (Zhang *et al.* (1998), Nucleic Acids Res. 26, 3986-3990), 3D-PSSM (Kelly *et al.* (2000) J. Mol. Biol. 299, 499-520); and other computational analysis methods (Shi *et al.* (1999) Biochem. Biophys. Res. Commun. 262, 132-138 and Matsunami *et al.* (2000) Nature 404, 601-604).

Recombinant DNA molecules Containing a Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain a coding sequence. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in situ*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.* (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press. In the preferred rDNA molecules, a coding DNA sequence is operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the protein family encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of

directing the replication or insertion into the host chromosome, and preferably also expression, of the structural gene included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

In one embodiment, the vector containing a coding nucleic acid molecule will include a prokaryotic replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or bacteriophage promoter capable of directing the expression (transcription and translation) of the coding gene sequences in a bacterial host cell, such as *E.coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 (BioRad), pPL and pKK223 (Pharmacia).

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form rDNA molecules that contain a coding sequence. Eukaryotic cell expression vectors, including viral vectors, are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase (*neo*) gene. (Southern *et al.* (1982) J. Mol. Anal. Genet. 1, 327-341). Alternatively, the selectable

marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker. The present invention further provides host cells transformed with a nucleic acid molecule that encodes a protein of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a protein of the invention are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human cell line. Preferred eukaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells (NIH-3T3) available from the ATCC as CRL 1658, baby hamster kidney cells (BHK), and the like eukaryotic tissue culture cell lines.

Any prokaryotic host can be used to express a rDNA molecule encoding a protein of the invention. The preferred prokaryotic host is *E.coli*.

Transformation of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.* (1972) Proc. Natl. Acad. Sci. USA 69, 2110; and Sambrook *et al.* (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press. With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.* (1973) Virol. 52, 456; Wigler *et al.* (1979) Proc. Natl. Acad. Sci. USA 76, 1373-1376.

Successfully transformed cells, *i.e.*, cells that contain a rDNA molecule of the present invention, can be identified by well known techniques including the selection for a selectable marker. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern (1975) J. Mol. Biol. 98, 503-504 or Berent *et al.* (1985) Biotech. 3, 208-209 or the proteins produced from the cell assayed via an immunological method.

Production of Recombinant Proteins using a rDNA Molecule

The present invention further provides methods for producing a protein of the invention using nucleic acid molecules herein described. In general terms, the production of a recombinant form of a protein typically involves the following steps:

A nucleic acid molecule is first obtained that encodes a protein of the invention, such as a nucleic acid molecule comprising, consisting essentially of or consisting of SEQ ID NO: 1 or nucleotides 251-4336 of SEQ ID NO: 1. If the encoding sequence is uninterrupted by introns, as is this open reading frame, it is directly suitable for expression in any host.

The nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the protein open reading frame. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the recombinant protein. Optionally the recombinant protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with the nucleic acid molecules of the invention to produce recombinant protein.

The Protein Associated with Bone Disorders

The present invention provides isolated proteins, allelic variants of the proteins, and conservative amino acid substitutions of the protein comprising the amino acids sequence of SEQ ID NO: 2. As used herein, the "protein" or "polypeptide" refers, in part, to a protein that has the human amino acid sequence depicted in SEQ ID NO: 2. The terms also refer to naturally occurring allelic variants and proteins that have a slightly different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the same or similar biological functions associated with these proteins.

As used herein, the family of proteins related to the human amino acid sequences of SEQ ID NO: 2 refers to proteins that have been isolated from organisms in addition to humans. The methods used to identify and isolate other members of the family of proteins related to these proteins are described below.

The proteins of the present invention are preferably in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated protein.

The proteins of the present invention further include insertion, deletion or conservative amino acid substitution variants of SEQ ID NO: 2. As used herein, a conservative variant refers to alterations in the amino acid sequence that does not adversely affect the biological functions of the protein. A substitution, insertion or deletion is said to adversely affect the protein when the altered sequence prevents or disrupts a biological function associated with the protein. For example, the overall charge, structure or hydrophobic/hydrophilic properties of the protein can be altered without adversely affecting a biological activity. Accordingly, the amino acid sequence can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the biological activities of the protein.

Ordinarily, the allelic variants, the conservative substitution variants, and the members of the protein family, will have an amino acid sequence having at least about 75% amino acid sequence identity with the entire sequence set forth in SEQ ID NO: 2, more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95% sequence identity. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Fusion proteins, or N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

Thus, the proteins of the present invention include molecules having the amino acid sequence disclosed in SEQ ID NO: 2 and fragments thereof having a consecutive sequence of at least about 1015 or more amino acid residues of these proteins; amino acid sequence variants wherein one or more amino acid residues has been inserted N- or C-terminal to, or within, the disclosed coding sequence; and amino acid sequence variants of the disclosed sequence, or their fragments as defined above, that have been substituted by at least one residue. Such fragments, also referred to as peptides or polypeptides, may contain antigenic regions, functional regions of the protein identified as regions of the amino acid sequence which correspond to known protein domains, as well as regions of pronounced hydrophilicity. The regions are all easily identifiable by using commonly available protein sequence analysis software such as MacVector (Oxford Molecular).

Contemplated variants further include those containing predetermined mutations by,

e.g., homologous recombination, site-directed or PCR mutagenesis, and the corresponding proteins of other animal species, including but not limited to rabbit, mouse, rat, porcine, bovine, ovine, equine and non-human primate species, and the alleles or other naturally occurring variants of the family of proteins; and derivatives wherein the protein has been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope).

The present invention further provides compositions comprising a protein or polypeptide of the invention and a diluent. Suitable diluents can be aqueous or non-aqueous solvents or a combination thereof, and can comprise additional components, for example water-soluble salts or glycerol, that contribute to the stability, solubility, activity, and/or storage of the protein or polypeptide.

As described below, members of the family of proteins can be used: (1) to identify agents which modulate the level of or at least one activity of the protein, (2) to identify binding partners for the protein, (3) as an antigen to raise polyclonal or monoclonal antibodies, (4) as a therapeutic agent or target and (5) as a diagnostic agent or marker of osteoporosis and other bone disorders.

Methods to Identify Binding Partners

Another embodiment of the present invention provides methods for use in isolating and identifying binding partners of proteins of the invention. In general, a protein of the invention is mixed with a potential binding partner or an extract or fraction of a cell under conditions that allow the association of potential binding partners with the protein of the invention. After mixing, peptides, polypeptides, proteins or other molecules that have become associated with a protein of the invention are separated from the mixture. The binding partner that bound to the protein of the invention can then be removed and further analyzed. To identify and isolate a binding partner, the entire protein, for instance a protein comprising the entire amino acid sequence of SEQ ID NO: 2 can be used. Alternatively, a fragment of the protein can be used.

As used herein, a cellular extract refers to a preparation or fraction that is made from a lysed or disrupted cell. The preferred source of cellular extracts will be cells derived from human skin tissue or the human respiratory tract or cells derived from a biopsy sample of human lung tissue in patients with allergic hypersensitivity. Alternatively, cellular extracts may be prepared from normal tissue or available cell lines, particularly granulocytic cell lines.

A variety of methods can be used to obtain an extract of a cell. Cells can be disrupted using either physical or chemical disruption methods. Examples of physical disruption methods

include, but are not limited to, sonication and mechanical shearing. Examples of chemical lysis methods include, but are not limited to, detergent lysis and enzyme lysis. A skilled artisan can readily adapt methods for preparing cellular extracts in order to obtain extracts for use in the present methods.

Once an extract of a cell is prepared, the extract is mixed with the protein of the invention under conditions in which association of the protein with the binding partner can occur. A variety of conditions can be used, the most preferred being conditions that closely resemble conditions found in the cytoplasm of a human cell. Features such as osmolarity, pH, temperature, and the concentration of cellular extract used, can be varied to optimize the association of the protein with the binding partner.

After mixing under appropriate conditions, the bound complex is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For example, antibodies specific to a protein of the invention can be used to immunoprecipitate the binding partner complex. Alternatively, standard chemical separation techniques such as chromatography and density/sediment centrifugation can be used.

After removal of non-associated cellular constituents found in the extract, the binding partner can be dissociated from the complex using conventional methods. For example, dissociation can be accomplished by altering the salt concentration or pH of the mixture. To aid in separating associated binding partner pairs from the mixed extract, the protein of the invention can be immobilized on a solid support. For example, the protein can be attached to a nitrocellulose matrix or acrylic beads. Attachment of the protein to a solid support aids in separating peptide/binding partner pairs from other constituents found in the extract. The identified binding partners can be either a single protein or a complex made up of two or more proteins. Alternatively, binding partners may be identified using a Far-Western assay according to the procedures of Takayama *et al.* (1997) Methods Mol. Biol. 69, 171-184 or Sauder *et al.* (1996) J. Gen.Virol. 77, 991-996 or identified through the use of epitope tagged proteins or GST fusion proteins.

Alternatively, the nucleic acid molecules of the invention can be used in a yeast two-hybrid system. The yeast two-hybrid system has been used to identify other protein partner pairs and can readily be adapted to employ the nucleic acid molecules herein described.

Modulation of Expression

The present inventors have identified the proteins of the invention as being associated with mesenchymal stem cell differentiation and subsequent osteoblast activity. Specifically, the expression and activation of the proteins of the invention, such as the protein having the amino acid sequence of SEQ ID NO: 2, in mesenchymal stem cells correlated with the maturation of

these cells into osteoblasts and subsequent deposition of bone. The present invention therefore includes methods for modulating expression and/or activity of the proteins of the invention to effect mesenchymal stem cell differentiation and osteoblast activity. Such methods will be useful in the treatment of disorders associated with abnormal osteoblast activity. Because osteoblast activity indirectly effects osteoclast activity via a general feedback mechanism, the invention also includes methods for modulating bone resorption associated with osteoclast activity.

Modulation of the gene, gene fragments, or the encoded protein of SEQ ID NO: 2 and fragments is useful in gene therapy to treat disorders associated with defects in the protein of the invention. In a preferred embodiment, expression is modulated to increase osteoblast activity in diseases with abnormal bone density. Expression vectors may be used to introduce the nucleic acids of the invention into a cell. Such vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences. Transcription cassettes may be prepared comprising a transcription initiation region, the target gene or fragment thereof, and a transcriptional termination region. The transcription cassettes may be introduced into a variety of vectors, *e.g.*, plasmid, retrovirus, lentivirus, adenovirus and the like, where the vectors are able to transiently or stably be maintained in the cells, usually for a period of at least about one day, more usually for a period of at least about several days to several weeks.

The proteins and nucleic acids of the invention may be introduced into tissues or host cells by any number of routes, including viral infection, microinjection, or fusion of vesicles. Jet injection may also be used for intramuscular administration, as described by Furth *et al.* (1992) Anal. Biochem. 205, 365-368. The DNA may be coated onto gold microparticles, and delivered intradermally by a particle bombardment device, or "gene gun" as described in the literature (see, for example, Tang *et al.* (1992) Nature 356, 152-154), where gold microprojectiles are coated with DNA, then bombarded into skin cells.

Antisense molecules can be used to down-regulate expression of nucleic acids or proteins of the invention in cells. The anti-sense reagent may be antisense oligonucleotides, particularly synthetic antisense oligonucleotides having chemical modifications from native nucleic acids, or nucleic acid constructs that express such anti-sense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene expression through various mechanisms, *e.g.*, by reducing the amount of mRNA available for translation, through activation of RNAse H or steric hindrance. One or a combination of antisense molecules may be administered, where a combination may comprise multiple different sequences.

Antisense molecules may be produced by expression of all or a part of the target gene sequence in an appropriate vector, where the transcriptional initiation is oriented such that an antisense strand is produced as an RNA molecule. Alternatively, the antisense molecule is a synthetic oligonucleotide. Antisense oligonucleotides will generally be at least about seven, usually at least about twelve, and more usually at least about twenty nucleotides in length. Typical antisense oligonucleotides are usually not more than about five-hundred, more usually not more than about fifty, and even more usually not more than about thirty-five nucleotides in length, where the length is governed by efficiency of inhibition, specificity, including absence of cross-reactivity, and the like. It has been found that short oligonucleotides, of from seven to eight bases in length, can be strong and selective inhibitors of gene expression (see Wagner *et al.* (1996) Nat. Biotech. 14, 840-844).

A specific region or regions of the endogenous sense strand mRNA sequence is chosen to be complemented by the antisense sequence. Selection of a specific sequence for the oligonucleotide may use an empirical method, where several candidate sequences are assayed for inhibition of expression of the target gene in an *in vitro* or animal model. A combination of sequences may also be used, where several regions of the mRNA sequence are selected for antisense complementation.

Antisense oligonucleotides may be chemically synthesized by methods known in the art (see Wagner *et al.* (1996) Nat. Biotech. 14, 840-844). Preferred oligonucleotides are chemically modified from the native phosphodiester structure, in order to increase their intracellular stability and binding affinity. A number of such modifications have been described in the literature, which alter the chemistry of the backbone, sugars or heterocyclic bases.

As an alternative to anti-sense inhibitors, catalytic nucleic acid compounds, *e.g.*, ribozymes, anti-sense conjugates, etc. may be used to inhibit gene expression. Ribozymes may be synthesized *in vitro* and administered to the patient, or may be encoded on an expression vector, from which the ribozyme is synthesized in the targeted cell (see, for example, WO 95/23225; Beigelman *et al.* (1995) Nucl. Acids Res. 23, 4434-4442). Examples of oligonucleotides with catalytic activity are described in WO 95/06764.

Methods to Identify Agents that Modulate Expression

Another embodiment of the present invention provides methods for identifying agents that modulate the expression of a nucleic acid encoding a protein of the invention such as a protein having the amino acid sequence of SEQ ID NO: 2. Such assays may utilize any available means of monitoring for changes in the expression level of the nucleic acids of the invention. As used herein, an agent is said to modulate the expression of a nucleic acid of the

invention if it is capable of up- or down-regulating expression of the nucleic acid in a cell.

In one assay format, cell lines that contain reporter gene fusions between the open reading frame defined by nucleotides 251-4,336 of SEQ ID NO: 1, or the 5' and/or 3' regulatory elements and any assayable fusion partner may be prepared. Numerous assayable fusion partners are known and readily available including the firefly luciferase gene and the gene encoding chloramphenicol acetyltransferase (Alam *et al.* (1990) Anal. Biochem. 188, 245-254). Cell lines containing the reporter gene fusions are then exposed to the agent to be tested under appropriate conditions and time. Differential expression of the reporter gene between samples exposed to the agent and control samples identifies agents that modulate the expression of a nucleic acid of the invention.

Additional assay formats may be used to monitor the ability of the agent to modulate the expression of a nucleic acid encoding a protein of the invention, such as the protein having SEQ ID NO: 2. For instance, mRNA expression may be monitored directly by hybridization to the nucleic acids of the invention. Cell lines are exposed to the agent to be tested under appropriate conditions and time and total RNA or mRNA is isolated by standard procedures such those disclosed in Sambrook *et al.* (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press).

Probes to detect differences in RNA expression levels between cells exposed to the agent and control cells may be prepared from the nucleic acids of the invention. It is preferable, but not necessary, to design probes which hybridize only with target nucleic acids under conditions of high stringency. Only highly complementary nucleic acid hybrids form under conditions of high stringency. Accordingly, the stringency of the assay conditions determines the amount of complementation that should exist between two nucleic acid strands in order to form a hybrid. Stringency should be chosen to maximize the difference in stability between the probe:target hybrid and probe:non-target hybrids.

Probes may be designed from the nucleic acids of the invention through methods known in the art. For instance, the G+C content of the probe and the probe length can affect probe binding to its target sequence. Methods to optimize probe specificity are commonly available in Sambrook *et al.* (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press or Ausubel *et al.* (1995) Current Protocols in Molecular Biology, Greene Publishing Co.

Hybridization conditions are modified using known methods, such as those described by Sambrook *et al.* and Ausubel *et al.* as required for each probe. Hybridization of total cellular RNA or RNA enriched for polyA RNA can be accomplished in any available format. For instance, total cellular RNA or RNA enriched for polyA RNA can be affixed to a solid support

and the solid support exposed to at least one probe comprising at least one, or part of one of the sequences of the invention under conditions in which the probe will specifically hybridize. Alternatively, nucleic acid fragments comprising at least one, or part of one of the sequences of the invention can be affixed to a solid support, such as a silicon chip or a porous glass wafer. The glass wafer can then be exposed to total cellular RNA or polyA RNA from a sample under conditions in which the affixed sequences will specifically hybridize. Such solid supports and hybridization methods are widely available, for example, those disclosed in WO 95/11755. By examining for the ability of a given probe to specifically hybridize to an RNA sample from an untreated cell population and from a cell population exposed to the agent, agents which up or down regulate the expression of a nucleic acid encoding the protein having the sequence of SEQ ID NO: 2 are identified.

Hybridization for qualitative and quantitative analysis of mRNA may also be carried out by using a RNase Protection Assay (*i.e.*, RPA, see Ma *et al.* (1996) Methods 10, 273-238). Briefly, an expression vehicle comprising cDNA encoding the gene product and a phage specific DNA dependent RNA polymerase promoter (*e.g.*, T7, T3 or SP6 RNA polymerase) is linearized at the 3' end of the cDNA molecule, downstream from the phage promoter, wherein such a linearized molecule is subsequently used as a template for synthesis of a labeled antisense transcript of the cDNA by *in vitro* transcription. The labeled transcript is then hybridized to a mixture of isolated RNA (*i.e.*, total or fractionated mRNA) by incubation at 45°C overnight in a buffer comprising 80% formamide, 40 mM Pipes (pH 6.4), 0.4 M NaCl and 1 mM EDTA. The resulting hybrids are then digested in a buffer comprising 40 μg/ml ribonuclease A and 2 μg/ml ribonuclease. After deactivation and extraction of extraneous proteins, the samples are loaded onto urea/polyacrylamide gels for analysis.

In another assay format, cells or cell lines are first identified which express the gene products of the invention physiologically. Cell and/or cell lines so identified would be expected to comprise the necessary cellular machinery such that the fidelity of modulation of the transcriptional apparatus is maintained with regard to exogenous contact of agent with appropriate surface transduction mechanisms and/or the cytosolic cascades. Further, such cells or cell lines would be transduced or transfected with an expression vehicle (*e.g.*, a plasmid or viral vector) construct comprising an operable non-translated 5'-promoter containing end of the structural gene encoding the instant gene products fused to one or more antigenic fragments, which are peculiar to the instant gene products, wherein said fragments are under the transcriptional control of said promoter and are expressed as polypeptides whose molecular weight can be distinguished from the naturally occurring polypeptides or may further comprise an immunologically distinct tag or other detectable marker. Such a process is well known in the

art (see Sambrook *et al.* (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press).

Cells or cell lines transduced or transfected as outlined above are then contacted with agents under appropriate conditions; for example, the agent in a pharmaceutically acceptable excipient is contacted with cells in an aqueous physiological buffer such as phosphate buffered saline (PBS) at physiological pH, Eagles balanced salt solution (BSS) at physiological pH, PBS or BSS comprising serum or conditioned media comprising PBS or BSS and/or serum incubated at 37°C. Said conditions may be modulated as deemed necessary by one of skill in the art. Subsequent to contacting the cells with the agent, said cells will be disrupted and the polypeptides of the lysate are fractionated such that a polypeptide fraction is pooled and contacted with an antibody to be further processed by immunological assay (*e.g.*, ELISA, immunoprecipitation or Western blot). The pool of proteins isolated from the "agent-contacted" sample will be compared with a control sample where only the excipient is contacted with the cells and an increase or decrease in the immunologically generated signal from the agent-contacted sample compared to the control will be used to distinguish the effectiveness of the agent.

Methods to Identify Agents that Modulate Activity

The present invention provides methods for identifying agents that modulate at least one activity of a protein of SEQ ID NO: 2. Such methods or assays may utilize any means of monitoring or detecting the desired activity.

In one format, the specific activity of a protein of the invention, normalized to a standard unit, between a cell population that has been exposed to the agent to be tested compared to an un-exposed control cell population may be assayed. Cell lines or populations are exposed to the agent to be tested under appropriate conditions and time. Cellular lysates may be prepared from the exposed cell line or population and a control, unexposed cell line or population. The cellular lysates are then analyzed with the probe.

Antibody probes can be prepared by immunizing suitable mammalian hosts utilizing appropriate immunization protocols using the proteins of the invention or antigen-containing fragments thereof. To enhance immunogenicity, these proteins or fragments can be conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as BSA, KLH or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co. may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy

terminus with a cysteine residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using standard methods, see *e.g.*, Kohler & Milstein (1992) Biotechnology 24, 524-526 or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies can be screened by immunoassay in which the antigen is the peptide hapten, polypeptide or protein. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies may be recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonal antibodies or the polyclonal antisera that contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as Fab or Fab' fragments, is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Antibody regions that bind specifically to the desired regions of the protein can also be produced in the context of chimeras with multiple species origin.

Antibody regions that bind specifically to the desired regions of the protein can also be produced in the context of chimeras with multiple species origin, for instance, humanized antibodies. The antibody can therefore be a humanized antibody or human a antibody, as described in U.S. Patent 5,585,089 or Riechmann *et al.* (1988) Nature 332, 323-327.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the a protein of the invention alone or with its associated substrates, binding partners, etc. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a non-random basis which takes into account the sequence of the target site or its

conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up these sites. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to or a derivative of any functional consensus site.

The agents of the present invention can be, as examples, peptides, peptide mimetics, antibodies, antibody fragments, small molecules, vitamin derivatives, as well as carbohydrates. Peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies or fragments thereof that bind to a protein of SEQ ID NO: 2. Antibody agents can be obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the protein intended to be targeted by the antibodies.

In yet another class of agents, the present invention includes peptide mimetics that mimic the three-dimensional structure of the protein of SEQ ID NO: 2. Such peptide mimetics may have significant advantages over naturally occurring peptides, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity and others.

In one form, mimetics are peptide-containing molecules that mimic elements of protein secondary structure. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

In another form, peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compounds are also referred to as peptide mimetics or peptidomimetics (Fauchere (1986) Adv. Drug Res. 15, 29-69; Veber & Freidinger (1985) Trends Neurosci. 8, 392-396; Evans *et al.* (1987) J. Med. Chem. 30, 1229-1239 which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling.

Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptide mimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biochemical

property or pharmacological activity), but have one or more peptide linkages optionally replaced by a linkage by methods known in the art.

Labeling of peptide mimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering positions on the peptide mimetic that are predicted by quantitative structure-activity data and molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules to which the peptide mimetic binds to produce the therapeutic effect. Derivitization (*e.g.*, labeling) of peptide mimetics should not substantially interfere with the desired biological or pharmacological activity of the peptide mimetic.

The use of peptide mimetics can be enhanced through the use of combinatorial chemistry to create drug libraries. The design of peptide mimetics can be aided by identifying amino acid mutations that increase or decrease binding of the protein to its binding partners. Approaches that can be used include the yeast two hybrid method (see Chien *et al.* (1991) Proc. Natl. Acad. Sci. USA 88, 9578-9582) and using the phage display method. The two hybrid method detects protein-protein interactions in yeast (Fields *et al.* (1989) Nature 340, 245-246). The phage display method detects the interaction between an immobilized protein and a protein that is expressed on the surface of phages such as lambda and M13 (Amberg *et al.* (1993) Strategies 6, 2-4; Hogrefe *et al.* (1993) Gene 128, 119-126). These methods allow positive and negative selection for protein-protein interactions and the identification of the sequences that determine these interactions.

Diagnostic Methods and Agents

As described above, expression of the proteins and nucleic acids of the invention may be used as a diagnostic marker for the prediction or identification of the differentiation state of a sample comprising precursor stem cells. In some embodiments, the tissue sample is a bone biopsy. For instance, a tissue sample may be assayed by any of the methods described above, and expression levels of the proteins or nucleic acids of the invention may be compared to the expression levels found in undifferentiated precursor stem cells and/or precursor stem cells induced to differentiate into osteoblasts and/or precursor stem cells induced to differentiate into a cell type other than an osteoblast. Such methods may be used to diagnose or identify conditions characterized by abnormal bone deposition, reabsorption and/or abnormal rates of osteoblast differentiation.

Those skilled in the art will appreciate that a wide variety of conditions are associated with abnormal bone deposition or loss. Such conditions include, but are not limited to, osteoporosis, osteopenia, osteodystrophy, and various other osteopathic conditions. The

methods of the present invention will be particularly useful in diagnosing or monitoring the treatment of conditions such as postmenopausal osteoporosis (PMO), glucocorticoid-induced osteoporosis (GIO), and male osteoporosis. Agents which modulate expression of the nucleic acids or proteins of the invention will be useful in treatment of these conditions.

In some preferred embodiments, the present invention may be used to diagnose and/or monitor the treatment of drug-induced abnormalities in bone formation or loss. For example, at present a combination of cyclosporine with prednisone is given to patients who have received an organ transplant in order to suppress tissue rejection. The combination causes rapid bone loss in a manner different than that observed with prednisone alone (such as elevated level of serum osteocalcin and vitamin D in patients treated with cyclosporine but not in patients treated with prednisone). Other drugs are also known to effect bone formation or loss. The anticonvulsant drugs diphenylhydantoin, phenobarbital and carbamazepine, and combination of these drugs, cause alterations in calcium metabolism. A decrease in bone density is observed in patients taking anticonvulsant drugs. Although heparin is an effective therapy for thromboembolic disorders, increased incidences of osteoporotic fractures have been reported in patients with heparin therapy hence the present invention will be useful to monitor patients undergoing heparin treatment.

Other embodiments of the present invention allow the diagnosis and/or monitoring of the treatment of other conditions that involve altered bone metabolism. For example, idiopathic juvenile osteoporosis (IJO) is a generalized decrease in mineralized bone in the absence of rickets or excessive bone resorption and typically occurs in children before the onset of puberty. In addition, thyroid diseases have been linked to bone loss. A decrease in bone mass has been shown in patients with thyrotoxicosis causing these individuals to be at increased risk of having fractures. These individuals also sustain fractures at an earlier age than individuals who have never been thyrotoxic.

Another situation in which the present invention will be useful is the diagnosis and/or monitoring of the treatment of skeletal disease linked to breast cancer. Breast cancer frequently metastasizes to the skeleton and about 70% of patients with advanced cancer develop symptomatic skeletal disease. Moreover, the anti-cancer treatments presently in use have been shown to lead to early menopause and bone loss when given to premenopausal women.

The present invention will be useful in diagnosing and/or monitoring the treatment of chronic anemia associated with abnormal bone formation or loss. Homozygous beta-thalassemia is usually described as an example of chronic anemia predisposing to osteoporosis. Patients with thalassemia have expansion of bone marrow space with thinning of the adjacent trabeculae.

Other conditions in which the present invention will find application are: Fanconi

syndrome where osteomalacia is a common feature; fibrous dysplasia, McCune-Albright syndrome refers to patients with fibrous dysplasia with a sporadic, developmental disorder characterized by a unifocal or multifocal expanding fibrous lesion of bone-forming mesenchyme that often results in pain, fracture or deformity; osteogenesis imperfecta (OI, also called brittle bone disease) is associated with recurrent fractures and skeletal deformity, various skeletal dysplasias *i.e.*, osteochondroplasia which is characterized by abnormal development of cartilage and/or bone and other diseases such as achodroplasia, mucopolysacchaidoses, dysostosis and ischemic bone diseases.

The present invention will be particularly useful by providing a marker that may be used as a marker of bone turnover to determine osteoporosis. The present invention may also be used *in vitro* in assays or treatments as a marker of osteoblast differentiation and proliferation.

Modulation of Gene Expression

As provided in the Examples, the proteins and nucleic acids of the invention are expressed on osteoblasts derived from mesenchymal stem cells. Agents that modulate or up- or down-regulate the expression of the protein or agents such as agonists or antagonists of at least one activity of the proteins of the invention may be used to modulate biological and pathologic processes associated with the protein's function and activity. The invention is particularly useful in the treatment of human subjects.

Pathological processes refer to a category of biological processes that produce a deleterious effect. For example, expression of the proteins of the invention is associated with differentiation of stem cells into osteoblasts under normal conditions but in a disease state, the necessary level of expression of the proteins may not be present. Such diseases include, but are not limited to, diseases caused by an abnormal rate of osteoblast formation and subsequent activity. Decreased osteoblast activity can lead to a decrease in bone deposition with a concurrent increased osteoclast activity resulting in abnormal increase in bone resorption ultimately leading to decreased bone density.

As discussed above, those skilled in the art will appreciate that a wide variety of conditions are associated with an abnormal rate of osteoblast formation leading to abnormal bone deposition or loss. Such conditions include, but are not limited to, osteoporosis, osteopenia, osteodystrophy, and various other osteopathic conditions. The methods of the present invention will be particularly useful in the treatment of conditions such as postmenopausal osteoporosis (PMO), glucocorticoid-induced osteoporosis (GIO), and male osteoporosis. Agents which modulate expression of the proteins of this invention will be useful in treatment of these conditions.

Osteoporosis is an example of one such disease characterized by abnormal bone density. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, a bone density disorder may be prevented or disease progression modulated by the administration of agents which reduce, promote or modulate in some way the expression or at least one activity of the proteins and nucleic acids of the invention, such as the protein having the amino acid sequence of SEQ ID NO: 2. For osteoporosis, the therapeutic strategy comprises a treatment with the agent until normal bone mass compared to appropriate control groups is restored. Bone mass can be assessed by determining bone mineral density. Then the treatment can be switched to established regimens for the prevention of bone loss to avoid potential side effects of overshooting bone formation.

Other embodiments of the present invention allow for the treatment of other conditions that involve altered bone metabolism associated with osteoblast activity, *e.g.*, idiopathic juvenile osteoporosis (IJO). In addition, thyroid diseases have been linked to bone loss. A decrease in bone mass has been shown in patients with thyrotoxicosis causing these individuals to be at increased risk of having fractures. These individuals also sustain fractures at an earlier age than individuals who have never been thyrotoxic.

The present invention will be useful in the treatment of abnormal bone formation or loss associated with chronic anemia. Homozygous beta-thalassemia is usually described as an example of chronic anemia predisposing to osteoporosis. Patients with thalassemia have expansion of bone marrow space with thinning of the adjacent trabeculae.

Other conditions in which the present invention will find therapeutic application are: Fanconi syndrome where osteomalacia is a common feature; fibrous dysplasia, McCune-Albright syndrome refers to patients with fibrous dysplasia with a sporadic, developmental disorder characterized by a unifocal or multifocal expanding fibrous lesion of bone-forming mesenchyme that often results in pain, fracture or deformity; osteogenesis imperfecta (OI, also called brittle bone disease) is associated with recurrent fractures and skeletal deformity, various skeletal dysplasias *i.e.*, osteochondroplasia which is characterized by abnormal development of cartilage and/or bone and other diseases such as achodroplasia, mucopolysacchaidoses, dysostosis and ischemic bone diseases.

In one example, administration of soluble form of the protein of the invention can be used to treat a bone density disorder associated with the proteins' expression. Soluble receptors have been used to bind cytokines or other ligands to regulate their function (Thomson (1998) Cytokine Handbook, Academic Press).

The agents of the present invention can be provided alone, or in combination, or in sequential combination with other agents that modulate a particular pathological process. As

used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time. For example, the agents of the invention can be used in combination with estrogen replacement therapy in postmenopausal osteoporosis.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. For example, an agent may be administered locally to a site of injury via microinfusion. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents that modulate expression or at least one activity of the proteins of the invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 1 pg/kg to 100 mg/kg body weight. The preferred dosages for systemic administration comprise 100 ng/kg to 100 mg/kg body weight. The preferred dosages for direct administration to a site via microinfusion comprise 1 ng/kg to 1 mg/kg body weight.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol and dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient. Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In practicing the methods of this invention, the agents of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be co-administered along with other compounds typically prescribed for these conditions according to generally accepted medical practice, such as anti-inflammatory agents, anticoagulants, antithrombotics, including platelet aggregation inhibitors, tissue plasminogen activators, urokinase, prourokinase, streptokinase, aspirin and heparin. The compounds of this invention can be utilized in vivo, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice, or *in vitro*.

Prognostic Uses

As described above, the nucleic acids and proteins of the invention and their expression may also be used as markers for the monitoring of disease progression, such as osteoporosis. For instance, a tissue sample may be assayed by any of the methods described above, and the expression levels for the protein may be compared to the expression levels found in undifferentiated precursor stem cells and/or precursor stem cells induced to differentiate into osteoblasts and/or precursor stem cells induced to differentiate into a cell type other than an osteoblast and/or osteoblasts.

Expression or activity the proteins and nucleic acids of the invention, such as the protein having the amino acid sequence of SEQ ID NO: 2, may also be used to track or predict the progress or efficacy of a treatment regime in a patient. For instance, a patient's progress or response to a given drug may be monitored by measuring gene expression of the proteins of the invention in a tissue or cell sample after treatment or administration of the drug. The expression of the protein in the post-treatment sample may then be compared to gene expression from undifferentiated precursor stem cells and/or precursor stem cells induced to differentiate into osteoblasts and/or precursor stem cells induced to differentiate into a cell type other than an osteoblast and/or osteoblasts and/or from tissue or cells from the same patient before treatment.

Transgenic Animals

Transgenic animals containing mutant, knock-out or modified genes corresponding to the cDNA sequence of SEQ ID NO: 1, or the open reading frame encoding the polypeptide sequence of SEQ ID NO: 2 or fragments thereof having a contiguous sequence of at least about one-thousand and fifteen amino acid residues, are also included in the invention. Transgenic animals are genetically modified animals into which recombinant, exogenous or cloned genetic material has been experimentally transferred. Such genetic material is often referred to as a

transgene. The nucleic acid sequence of the transgene, in this case a form of SEQ ID NO: 1 may be integrated either at a locus of a genome where that particular nucleic acid sequence is not otherwise normally found or at the normal locus for the transgene. The transgene may consist of nucleic acid sequences derived from the genome of the same species or of a different species than the species of the target animal.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic alteration or genetic information was introduced into a germ line cell, thereby conferring the ability of the transgenic animal to transfer the genetic information to offspring. If such offspring in fact possess some or all of that alteration or genetic information, then they too are transgenic animals.

The alteration or genetic information may be foreign to the species of animal to which the recipient belongs, foreign only to the particular individual recipient, or may be genetic information already possessed by the recipient. In the last case, the altered or introduced gene may be expressed differently than the native gene.

Transgenic animals can be produced by a variety of different methods including transfection, electroporation, microinjection, gene targeting in embryonic stem cells and recombinant viral and retroviral infection (see, *e.g.*, U.S. Patents 4,736,866 & 5,602,307; Mullins *et al.* (1993) Hypertension 22, 630-633; Brenin *et al.* (1997) Surg. Oncol. 6, 99-110; Tuan (1997) Recombinant Gene Expression Protocols, Methods in Molecular Biology, Humana Press).

A number of recombinant or transgenic mice have been produced, including those which express an activated oncogene sequence (U.S. Patent 4,736,866); express simian SV40 T-antigen (U.S. Patent 5,728,915); lack the expression of interferon regulatory factor 1 (IRF-1) (U.S. Patent 5,731,490); exhibit dopaminergic dysfunction (U.S. Patent 5,723,719); express at least one human gene which participates in blood pressure control (U.S. Patent 5,731,489); display greater similarity to the conditions existing in naturally occurring Alzheimer's disease (U.S. Patent 5,720,936); have a reduced capacity to mediate cellular adhesion (U.S. Patent 5,602,307); possess a bovine growth hormone gene (Clutter *et al.* (1996) Genetics 143, 1753-1760); or are capable of generating a fully human antibody response (McCarthy (1997) Lancet 349, 405-406).

While mice and rats remain the animals of choice for most transgenic experimentation, in some instances it is preferable or even necessary to use alternative animal species. Transgenic procedures have been successfully utilized in a variety of non-murine animals, including sheep, goats, pigs, dogs, cats, monkeys, chimpanzees, hamsters, rabbits, cows and guinea pigs (see, *e.g.*, Kim *et al.* (1997) Mol. Reprod. Dev. 46, 515-526; Houdebine (1995) Reprod. Nutr. Dev.

35, 609-617; Petters (1994) Reprod. Fertil. Dev. 6, 643-645; Schnieke *et al.* (1997) Science 278, 2130-2133; and Amoah (1997) J. Animal Science 75, 578-585).

The method of introduction of nucleic acid fragments into recombination competent mammalian cells can be by any method that favors co-transformation of multiple nucleic acid molecules. Detailed procedures for producing transgenic animals are readily available to one skilled in the art, including the disclosures in U.S. Patents 5,489,743 & 5,602,307.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1

Cloning of Full Length Human Gene

The full length cDNA having SEQ ID NO: 1 was obtained by the solution hybridization method. Briefly, a gene-specific oligonucleotide was designed based on the sequence of an EST fragment identified by READS analysis (Prashar *et al.* (1996) 93, 659-663). The oligonucleotide was labeled with biotin and used to hybridize with 5 μg of single strand plasmid DNA (cDNA recombinants) from a human resting mast cell library following the procedures from the Gene Trapper kit (Life Technologies). The hybridized cDNA was separated by streptavidin-conjugated beads and eluted by Tris-EDTA buffer. The eluted cDNA was converted to double strand plasmid DNA and used to transform *E. coli* cells (DH5α). Clones were screened by PCR using gene specific primers designed from the EST sequence to identify positive clones. After positive selection, the cDNA clone was subjected to DNA sequence.

The nucleotide sequences of the full-length human cDNA corresponding to the differentially regulated mRNA detected above is set forth in SEQ ID NO: 1. The cDNA comprises 7,084 base pairs, with an open reading frame at nucleotides 251-4,336 encoding a protein of 1,361 amino acids (nucleotides 251-4,335 without the TAA stop codon). The amino acid sequence corresponding to the encoded protein is set forth in SEQ ID NO: 2. Figure 6 displays the results of a hydrophobicity analysis of the polypeptide of SEQ ID NO: 2 using the methods of Kyte & Doolottle (1982) *J. Mol. Biol.* 157, 105-132.

Example 2

Down-regulation of Expression in hFSC

Human Fetal Stromal Cells (hFSC) were isolated from the bone marrow of a twenty-week human embryo. hFSCs are derived from a primary culture and represent a heterogeneous population of osteoprogenitor cells. hFSCs exhibit a high replicative capacity, with a doubling time of approximately twenty hours. hFSCs retain a spindle-shaped morphology and have a uniform attachment throughout subcultivation. hFSCs can be sub-cultured up to twelve passages while retaining both proliferative and osteogenic capability.

hFSCs used for READS analysis or Q-PCR were cultured in Dulbecco's Modified Eagle Medium (DMEM)-high glucose or DMEM-low glucose plus 10% fetal bovine serum, respectively, at 37°C in a humidified atmosphere containing 95% air and 5% carbon dioxide in the absence and presence of the indicated treatment. RNA was extracted from the cells at thirty minutes, three hours, six hours, twelve hours, twenty-four hours, forty-eight hours, three days, six days, twelve days and twenty-four days. When indicated, cells were contacted with either bone morphogenic protein-2 (BMP-2) at 300 ng/ml or transforming growth factor beta (TGF-β) at 1 ng/ml. Cells were incubated for the period of time indicated and harvested.

Total cellular RNA was prepared from the human fetal stromal cells described above. Synthesis of cDNA was performed as previously described in WO 97/05286 and in Prashar *et al.* (1996) Proc. Natl. Acad. Sci. USA 93, 659-663. Briefly, cDNA was synthesized according to the protocol described in the Gibco-BRL kit for cDNA synthesis. The reaction mixture for first-strand synthesis included 0.006 mg of total RNA, and 200 ng of a mixture of one-base anchored oligo(dT) primers with all three possible anchored bases (acgtaatacgactcactatagggcgaattgggtcgact$_{17}$n1 wherein n1 = a, c or g) (SEQ ID NO: 3) along with other components for first-strand synthesis reaction except reverse transcriptase. This mixture was incubated at 65°C for five minutes, chilled on ice and the process repeated.

Alternatively, the reaction mixture may include 0.010 mg of total RNA, and 2 pmol of one of the two base anchored oligo(dT) primers annealed such as RP5 (ctctcaaggatcttaccgctt$_{18}$at) (SEQ ID NO: 4), RP6 (taataccgcgccacatagcat$_{18}$cg) (SEQ ID NO: 5) or RP92 (cagggtagacgacgctacgct$_{18}$ga) (SEQ ID NO: 6) along with other components for first-strand synthesis reaction except reverse transcriptase. This mixture was then layered with mineral oil and incubated at 65°C for seven minutes followed by 50°C for another seven minutes. At this stage, 0.002 ml of Superscript® reverse transcriptase (Gibco-BRL) (200 units per microliter) was added quickly and mixed, and the reaction continued for one hour at 45-50°C. Second-strand synthesis was performed at 16°C for two hours. At the end of the reaction, the cDNA were precipitated with ethanol and the yield of cDNA was calculated. In these experiments, 200 ng of cDNA was obtained from 0.010 mg of total RNA. The adapter oligonucleotide sequences were

A1 (tagcgtccggcgcagcgacggccag) (SEQ ID NO: 7) and A2 (gatcctggccgtcggctgtctgtcggcgc) (SEQ ID NO: 8).

One microgram of oligonucleotide A2 was first phosphorylated at the 5' end using T4 polynucleotide kinase (PNK). After phosphorylation, PNK was heated denatured and 0.001 mg of the oligonucleotide A1 was added along with 10× annealing buffer (1 M NaCl/100 mM Tris-HCl (pH 8.0)/10 mM EDTA (pH 8.0)) in a final volume of 0.020 ml. This mixture was then heated at 65°C for ten minutes followed by slow cooling to room temperature for thirty minutes, resulting in formation of the Y adapter at a final concentration of 100 ng per microliter. About 20 ng of the cDNA was digested with four units of BglII in a final volume of 0.01 ml for thirty minutes at 37°C. Two microliters (4 ng of digested cDNA) of this reaction mixture was then used for ligation to 100 ng (fifty-fold) of the Y-shaped adapter in a final volume of 0.005 ml for sixteen hours at 15°C. After ligation, the reaction mixture was diluted with water to a final volume of 0.080 ml (adapter ligated cDNA concentration, 0.05 ng/ml) and heated at 65°C for ten minutes to denature T4 DNA ligase and 0.002 ml aliquots (with 100 pg of cDNA) were used for PCR.

The following sets of primers were used for PCR amplification of the adapter ligated 3'-end cDNA: tgaagccgagacgtcggtcg$(t)_{18}$ n1, n2 (SEQ ID NO: 9) (wherein n1, n2 = aa, ac, ag, at, ca, cc, cg, ct, ga, gc, gg and gt) as the 3' primer with A1 as the 5' primer or alternatively P5, RP6 or RP92 used as 3' primers with primer A1.1 serving as the 5' primer. To detect the PCR products on the display gel, 24 pmol of oligonucleotide A1 or A11 was 5'-end labeled using 0.015 ml of gamma-[^{32}P]ATP (Amersham; 3000 Ci/mmol) and PNK in a final volume of 0.020 ml for thirty minutes at 37°C. After heat denaturing PNK at 65°C for twenty minutes, the labeled oligonucleotide was diluted to a final concentration of 0.002 mM in 0.080 ml with unlabeled oligonucleotide A11. The PCR mixture (0.020 ml) consisted of 0.002 ml (100 pg) of the template, 0.002 ml of 10× PCR buffer (100 mM Tris·HCl (pH 8.3)/500 mM KCl), 0.002 ml of 15 mM magnesium chloride to yield 1.5 mM final magnesium concentration optimum in the reaction mixture, 0.20 mM dNTPs, 200 nM each 5' and 3' PCR primers, and one unit of Amplitaq Gold® DNA polymerase.

Primers and dNTPs were added after preheating the reaction mixture containing the rest of the components at 85°C. This "hot start" PCR was done to avoid amplification artifacts arising out of arbitrary annealing of PCR primers at lower temperature during transition from room temperature to 94°C in the first PCR cycle. PCR consisted of five cycles of 94°C for thirty seconds, 55°C for two minutes and 72°C for sixty seconds followed by twenty-five cycles of 94°C for thirty seconds, 60°C for two minutes, and 72°C for sixty seconds. A higher number of cycles resulted in smeary gel patterns. PCR products (0.0025 ml) were analyzed on 6%

polyacrylamide sequencing gel. For double or multiple digestion following adapter ligation, 0.0132 ml of the ligated cDNA sample was digested with a secondary restriction enzymes in a final volume of 0.020 ml. From this solution, 0.003 ml was used as template for PCR. This template volume of carried 100 pg of the cDNA and 10 mM magnesium chloride (from the 10× enzyme buffer), which diluted to the optimum of 1.5 mM in the final PCR volume of 0.020 ml. Since magnesium comes from the restriction enzyme buffer, it was not included in the reaction mixture when amplifying secondarily cut cDNA.

Individual cDNA fragments corresponding to nucleic acid molecules of SEQ ID NO: 1 were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Bands identified as having different expression levels in treated versus untreated human fetal stromal cells were extracted from the display gels as described by Liang *et al.* (1995) Curr. Opin. Immunol. 7, 274-280), reamplified using the 5' and 3' primers, and subcloned into PCR-Script with high efficiency using the PCR-Script® cloning kit (Stratagene). Plasmids were sequenced by cycle sequencing on an ABI automated sequencer. Alternatively, bands were extracted (cored) from the display gels, PCR amplified and sequenced directly without subcloning.

Figures 1A and B present a graphic depiction of the expression level of the target mRNA of SEQ ID NO: 1 whose expression pattern was found to be dependent upon the activation state of the precursor stem cells. These figures represent the data obtained from READS gel analysis of the mRNA expression data from hFSC. READS analysis (as described above) was performed on total RNA samples isolated from hFSC that were treated with TGF-β (1 ng/ml of culture media) for twenty-four days in Figure 1A and forty-eight hours in Figure 1B. Time points for Figure 1A were selected at one, three, six, twelve and twenty-four days post-initial treatment. Time points for Figure 1B were selected at three, six, twelve, twenty-four and forty-eight hours.

Figures 2A and B present a graphic depiction of the expression level of the target mRNA of SEQ ID NO: 1. READS analysis (as described above) was performed on total RNA samples isolated from hFSC that were treated with BMP-2 (300 ng/ml of culture media) for twenty-four days in Figure 2A and forty-eight hours in Figure 2B. Time points for Figure 2A were selected at one, three, six, twelve and twenty-four days post-initial treatment. Time points for Figure 2B were selected at three, six, twelve, twenty-four and forty-eight hours.

Figures 3A, 3B and 3C provides a graphical representation of the expression level of the target mRNA of SEQ ID NO: 1 in human mesenchymal stem cells as assayed using READS gel analysis in response to treatment with osteogenic and adipogenic agents. In Figure 3A, cells were cultured in a medium supplemented with 10% fetal calf serum with or without

dexamethasone for time period ranging from zero to seven days). In Figure 3B, cells were cultured in the same manner with or without BMP-2 for the same time period. In Figure 3C, cells were cultured in a medium containing 10% rabbit serum with or without addition of dexamethasone for the same time period.

Control cells received media only with no added osteogenic agent or adipogenic agent. Subsequent to READS gel analysis, the images of each gel were converted into electronic format and the intensities of each band of interest were calculated relative to the background autoradiographic intensity of each gel image. The corrected values are termed adjusted intensity values, which were plotted on the y-axis versus the time course of the experiment.

Example 3

Quantitative RT-PCR analysis of Expression in hFSC and hMSC

Both human fetal stromal cells (hFSC) and hMSC were used for this study as in the READS experiments. Briefly, PCR primers and TaqMan probes were designed using the DNA sequences provided by sequence analysis of the nucleic acid molecule of SEQ ID NO: 1. Experimental conditions were as follows: hFSC were cultured *in vitro* and were left untreated for up to twenty-four days, or were treated with the osteogenic agents TGF-β (1 ng/ml of culture media) or BMP-2 (300 ng/ml) for the same time period.

Cells in each of the treatment groups were harvested at various time points after addition of TGF-β or BMP-2. Total RNA was isolated from the cells using Trizol® and the RNA was quantitated using a spectrophotometer set at 260 nm. Ten ng of total RNA was assayed in duplicate using the TaqMan® assay (Perkin-Elmer) in biplex format where each target gene in each RNA sample was assayed versus a reference mRNA which was shown previously to be constitutively expressed and not regulated by any of the osteogenic treatments. The Ct values of the target and reference gene were analyzed and the delta Ct values were calculated for each RNA sample. Fold change (expressed as relative expression) was plotted versus the time course of the experiment. Expression was relative to the delta Ct value (Target Ct minus Reference Ct) for t = 0 which was set to a value of 1.0.

Figure 4 shows expression levels of the target mRNA of SEQ ID NO: 1 in human fetal stromal cells (A and B) and in human mesenchymal stromal cells (C) as assayed by quantitative RT-PCR. In Figure 4A, cells were cultured using non-mineralization conditions in the absence or presence of either 1 ng/ml TGF-β1 or 300 ng/ml of BMP-2 (closed triangles) for time periods up to six days. In Figure 4B, cells were cultured using mineralization conditions in the absence of the same agents of Figure 4A for time periods up to twenty-one days. In Figure 4C, mesenchymal stromal cells were cultured in the presence of ascorbic acid and β-

glycerophosphate in the absence and presence of either TGF-β1, BMP-2 or dexamethasone for time periods up to sixteen days.

Example 4

Expression in human tissues

The tissue distribution of mRNA encoding the 76032 gene (SEQ ID NO: 1) was analyzed by quantitative PCR expression analysis of RNA isolated from various tissues. RNA was isolated from human kidney, spinal cord, adrenal gland, adipose tissue, heart, skeletal tissue, colon, pancreas, liver, prostate, thyroid, brain, stomach, small intestine, bone marrow, thymus, spleen, lung, uterus, mammary gland and trachea using standard procedures. PCR expression analysis was also performed using primers derived from the 76032 sequence using AmpliTaq® PCR amplification kits (Perkin Elmer). The presence of variable levels of mRNA encoding SEQ ID NO: 2 was detected in several tissues other than hFSC and hMSC (Figure 5). mRNA expression was most abundant in the spinal chord, adipose tissue and prostate. Detectable lower levels were observed in all other tissues tested.

Figure 5 shows expression levels, depicted as Ct values, of the target mRNA of SEQ ID NO: 1 in various human tissues as assayed using TaqMan quantitative RT-PCR methods (described above). The Ct values are displayed on the y axis whereas the tissue panel utilized in the assay is provided on the x axis. Expression levels of the target mRNA in resting human fetal stromal cells (HFSC control) and human mesenchymal stem cells (MSC control) is also provided

Example 5

Northern Blot Analysis

Figures 7 and 8 show a Northern blot in which the expression level of SEQ ID NO: 1 was measured in several normal human tissues including brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung and in leukocytes (ClonTech human mRNA blot-H12) as well as induced stem cells. RNA markers are present on the left side of the blot. Briefly, a cDNA clone corresponding to SEQ ID NO: 1, was radiolabeled using random primer labeling technology and the resulting probe was hybridized onto the blot during a sixteen hour incubation at 42°C in a 50% formamide hybridization solution. After the hybridization, the blot was washed in 0.1× SSC, 0.1% SDS at 42°C for up to two hours. After washing, the blot was exposed to film for a period of twenty-four hours at –80°C prior to development to obtain the figure shown. Figure 8 shows a Northern blot in which the expression level of SEQ ID NO: 1 was measured in human tissues as well as in human fetal stromal cells (FSC) and

mesenchymal stem cells (MSC) either resting or treated with osteogenic agents. A radiolabeled probe corresponding to SEQ ID NO: 1 was constructed as described above and was hybridized onto the blot in Church-Gilbert solution for sixteen hours at 65°C. After hybridization, the blot was washed in 0.5× SSC, 0.1% SDS at room temperature for two hours before exposure to film. The resulting autorad is shown in the Figure 8.

Example 6

Drug Screening Assays

Candidate agents and compounds will be screened for their ability to modulate the expression levels and/or activities of the gene comprising SEQ ID NO: 1 and identified as being involved in the differentiation of precursor stem cells into osteoblasts by any technique known to those skilled in the art including those assays described above. In some preferred embodiments, the assay of gene expression level may be conducted using real time PCR. Real time PCR detection may be accomplished by the use of the ABI Prism 7700 Sequence Detection System. The 7700 measures the fluorescence intensity of the sample each cycle and is able to detect the presence of specific amplicons within the PCR reaction. Each sample is assayed for the level of 76032 gene expression identified as being involved in the differentiation of precursor cells into osteoblasts.

The expression level of a control gene, for example GAPDH, may be used to normalize the expression levels. Suitable primers for the candidate genes may be selected using techniques well known to those skilled in the art. These primers may be used in conjunction with SYBR green (Molecular Probes), a nonspecific double stranded DNA dye, to measure the expression level mRNA corresponding to the 76032 gene, which will typically be normalized to the GAPDH level in each sample.

Normalized expression levels from cells exposed to the agent are then compared to the normalized expression levels in control cells. Agents that modulate the expression of the protein of this invention may be further tested as drug candidates in appropriate *in vitro* and *in vivo* models.

Example 7

Inhibition of 76032 gene expression increases osteoblast differentiation

Human mesenchymal stromal cells (hMSCs) were used for this study in addition to short interfering RNA (siRNA) designed to inhibit the expression of mRNA transcripts for the 76032 gene. siRNA effects were controlled using a control siRNA duplex containing an identical combination of bases which does not affect 76032 expression. Each well of a 48 well

plate was incubated with 0.1nM siRNA 0.005 mg Lipofectamine® 2000 (Invitrogen) for fifteen minutes. The total volume of this solution was then made up to 250 µl with culture medium and added to the well. Sufficient BMP-2, ascorbic acid and beta-glycerophosphate was then added to each well to give concentrations of 100 ng/ml, 50 µM and 10mM, respectively. Cells in each of the treatment groups were harvested 96 hours after addition of siRNA duplexes and BMP-2. Total RNA was isolated from the cells using Trizol® and the RNA was quantitated using a spectrophotometer set at 260 nm. 50 ng of total RNA was assayed in duplicate using the TaqMan® assay (Perkin-Elmer) in singleplex format where each target gene in each RNA sample was assayed versus a reference mRNA which was shown previously to be constitutively expressed and not regulated by any of the osteogenic treatments. The Ct values of the target and reference gene were analyzed and the delta Ct values were calculated for each RNA sample. Fold change (expressed as relative expression) was plotted versus cell treatment. For measurement of 76032 transcripts expression was relative to the delta Ct value (Target Ct minus Reference Ct) for control duplex treated cells. For measurement of alkaline phosphatase expression was relative to BMP-2 control cells.

Figure 9A shows expression levels of alkaline phosphatase mRNA, a marker of osteoblast differentiation, in human mesenchymal stromal cells treated with BMP-2 or in combination with siRNA duplex or control duplex as assayed by quantitative RT-PCR. Expression of alkaline phosphatase, and therefore osteoblast differentiation, was significantly increased by treatment of cells with the siRNA duplex. Figure 9B shows expression levels of the target mRNA (76032) in the same human mesenchymal stromal cells, demonstrating significant inhibition of the target gene by the siRNA duplex.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, patent applications and publications referred to in this application are herein incorporated by reference in their entirety.

We claim:

1. An isolated nucleic acid molecule selected from the group consisting of: (a) an isolated nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1; (b) an isolated nucleic acid molecule encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 2; (c) an isolated nucleic acid molecule that encodes a polypeptide fragment of at least about 1,015 amino acids of SEQ ID NO: 2; and (d) an isolated nucleic acid molecule that encodes a polypeptide that exhibits at least about 75% amino acid sequence identity to SEQ ID NO: 2.

2. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule comprises nucleotides 251-4,336 of SEQ ID NO: 1.

3. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule consists of nucleotides 251-4,336 of SEQ ID NO: 1.

4. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule comprises nucleotides 251-4333 of SEQ ID NO: 1.

5. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule consists of nucleotides 251-4333 of SEQ ID NO: 1.

6. The isolated nucleic acid molecule of any one of claims 1-5, wherein said nucleic acid molecule is operably linked to one or more expression control elements.

7. A vector comprising an isolated nucleic acid molecule of any one of claims 1-5.

8. A host cell transformed to contain the nucleic acid molecule of any one of claims 1-5.

9. A host cell comprising the vector of claim 8.

10. The host cell of claim 9, wherein said host is selected from the group consisting of prokaryotic host cells and eukaryotic host cells.

11. A method for producing a polypeptide comprising culturing a host cell transformed

with the nucleic acid molecule of any one of claims 1-5 under conditions in which the polypeptide encoded by said nucleic acid molecule is expressed.

12. The method of claim 11, wherein said host cell is selected from the group consisting of prokaryotic host cells and eukaryotic host cells.

13. An isolated polypeptide produced by the method of claim 11.

14. An isolated polypeptide selected from the group consisting of: (a) an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 2; (b) an isolated polypeptide comprising a fragment of at least 1015 amino acids of SEQ ID NO: 2; (c) an isolated polypeptide comprising conservative amino acid substitutions of SEQ ID NO: 2; and (d) an isolated polypeptide exhibiting at least about 75% amino acid sequence identity with SEQ ID NO: 2.

15. The isolated polypeptide of claim 15, wherein the polypeptide comprises SEQ ID NO: 2.

16. An isolated antibody that binds to a polypeptide of either claim 14 or 15.

17. An antibody of claim 16 wherein said antibody is a monoclonal or a polyclonal antibody.

18. A method of screening for an agent that modulates the differentiation of a population of stem cells into osteoblast cells comprising:

(a) exposing a population of stem cells to the agent, and

(b) measuring expression or activity of a nucleic acid molecule of claim 1 or a polypeptide encoded by the nucleic acid of claim 1 following exposure to the agent, wherein an decrease in the level of expression or activity is indicative of an agent capable of stimulating stem cells to differentiate into osteoblast cells.

19. A method of screening for an agent that increases bone density comprising:

(a) exposing a population of stem cells to the agent; and

(b) measuring expression or activity of a nucleic acid molecule of claim 1 or a polypeptide encoded by the nucleic acid of claim 1 following exposure to the agent, wherein a

decrease in the level of expression or activity is indicative of an agent capable increasing bone density.

20. A method of diagnosing a condition characterized by abnormal stem cell differentiation comprising detecting in a stem cell sample the level of expression or activity of a nucleic acid molecule of claim 1 or a polypeptide encoded by the nucleic acid of claim 1, wherein abnormal expression or activity is indicative of a condition characterized by abnormal stem cell differentiation.

21. A method of diagnosing a condition characterized by abnormal bone density comprising detecting in a stem cell sample the level of expression or activity of a nucleic acid molecule of claim 1 or a polypeptide encoded by the nucleic acid of claim 1, wherein a decrease in expression or activity is indicative of a condition characterized by abnormal bone density.

22. The method of claim 20 or 21 wherein the condition is osteoporosis.

23. A non-human transgenic animal comprising a nucleic acid molecule of claim 1.

24. A non-human transgenic animal that is engineered to not express a protein encoded by a nucleic acid molecule of claim 1.

Figure 1A

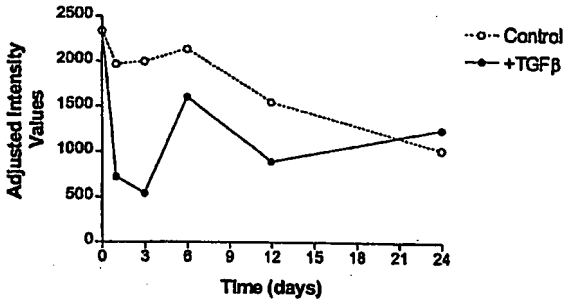


Figure 1B

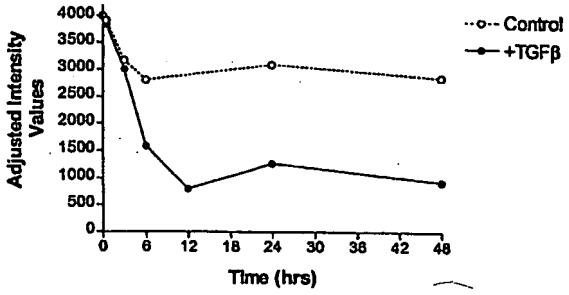


Figure 2A

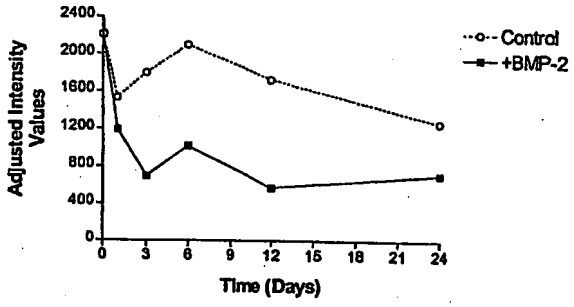


Figure 2B

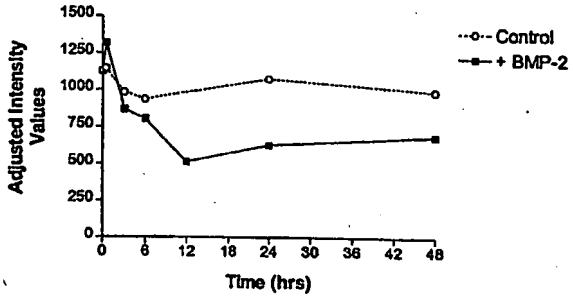
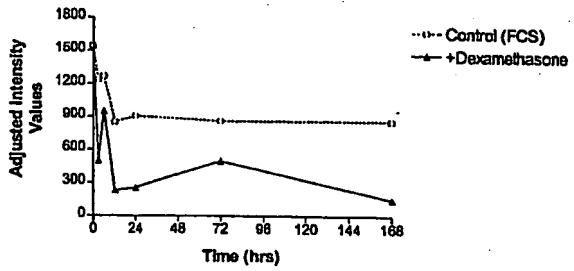


Figure 3A



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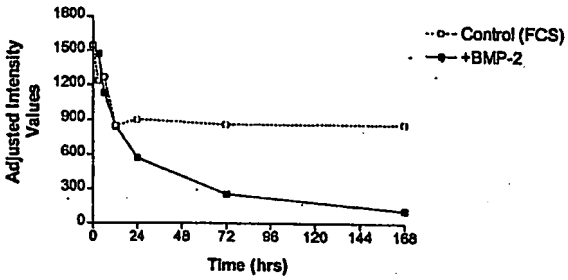

Figure 3C

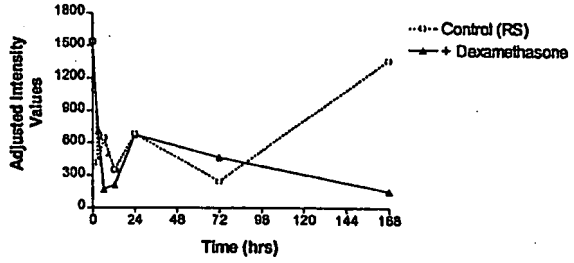

Figure 4

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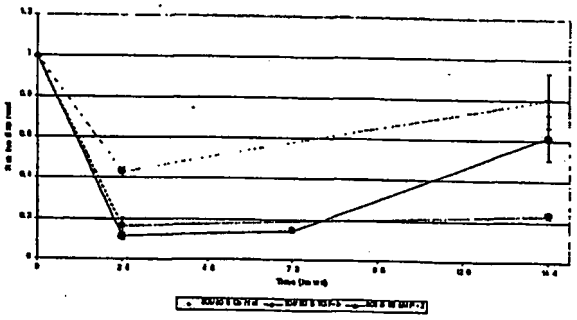

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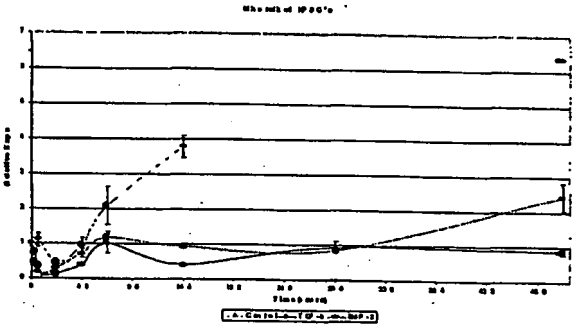

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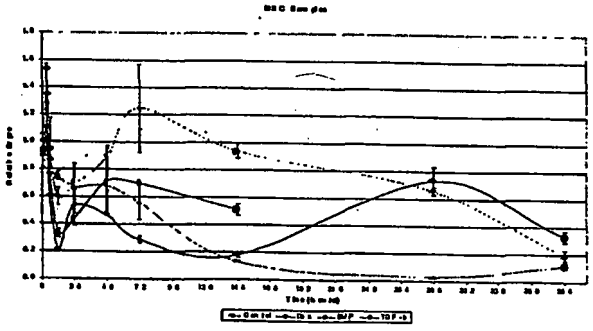


Figure 5: Human Tissue Panel Expression

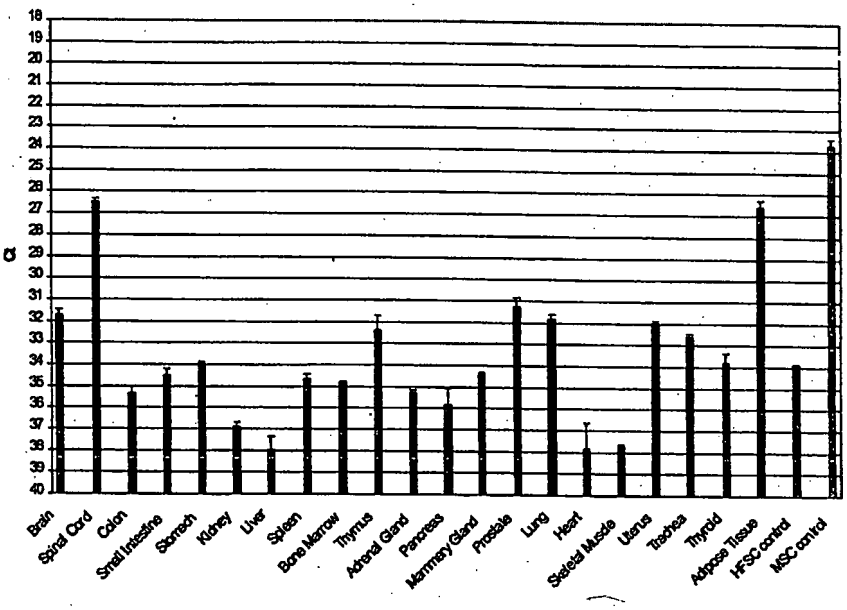
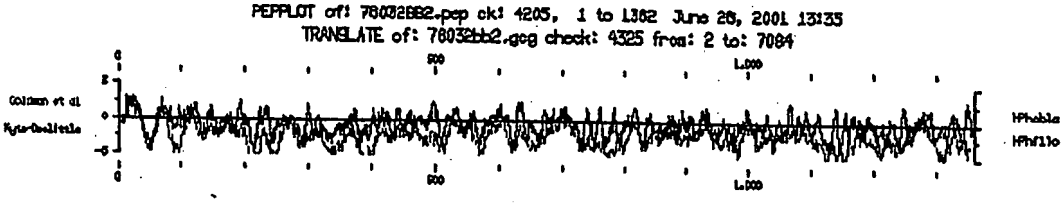


Figure 6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Goldman et al
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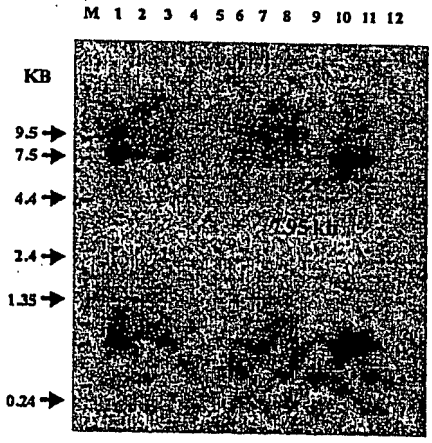
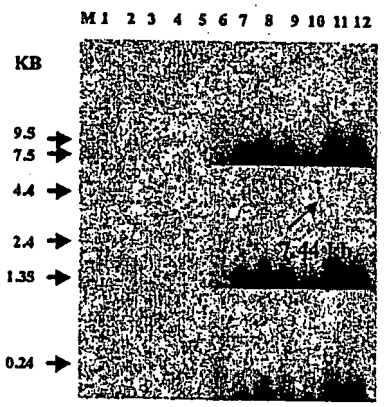
Figure 7: Northern Analysis of Human Tissue Panel



Blot: ClonTech Human mRNA blot-H12.
Probe: PGO-76032-BB2
(Random Labeling Method)
Hybridization buffer: Ultrahyb
Hybridization time: o/n at 42 °C.
Exposure: 24 hours at -80 °C.
Wash condition: until 0.1 x SSC
0.1% SDS at 42 °C.

M: RNA marker; 1. Brain; 2. Heart; 3. S. muscle;
4. Colon; 5. Thymus; 6. Spleen; 7. Kidney; 8. Liver
9. S. Intestine; 10. Placenta; 11. Lung; 12. Leukocyte

Figure 8: Northern Analysis of Human Stem Cell Samples



Blot: PGO Custom Northern (010507)
Probe: PGO-76032-BB2
(Random Labelling)
Hybridization buffer: Church-Gilbert
Hybridization time: o/n at 65 °C.
Exposure: 20 hours at -80 °C.
Final Wash condition: 0.5 x SSC
0.1% SDS
at RT

M: RNA marker; 1. Lung (BioChain); 2. Liver (BioChain); 3. Colon (BioChain); 4. Colon (Ambion); 5. Kidney (Clontech); 6. FSC; 7. NHOstRNA(S04462A); 8. MSC 24 h BMP-2+AB (S04546A); 9. MSC 24 h BMP-2 (S04545A); 10. MSC 24 h DEX (S04544A); 11. MSC 24 h AB (S04543A); 12. MSC 24 h Control (S04542A)

Figure 9

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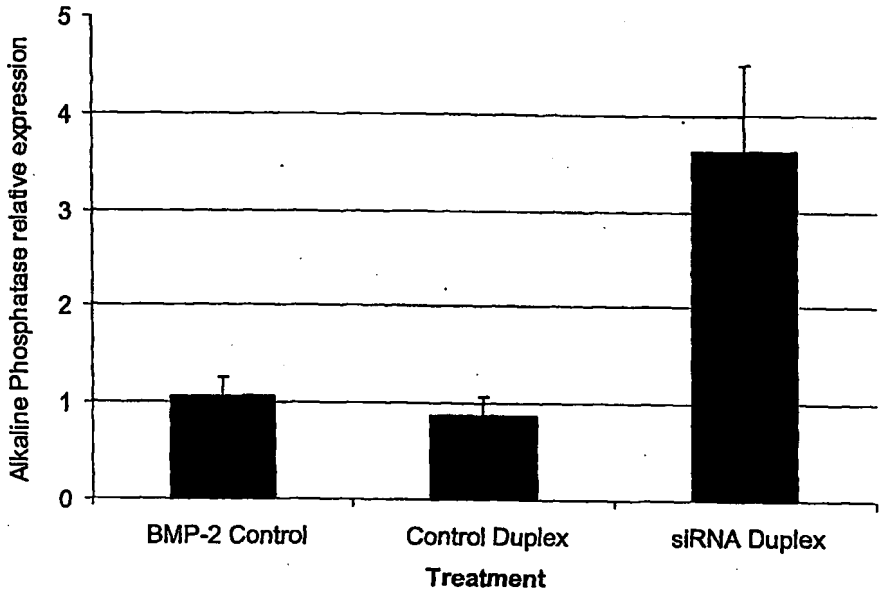
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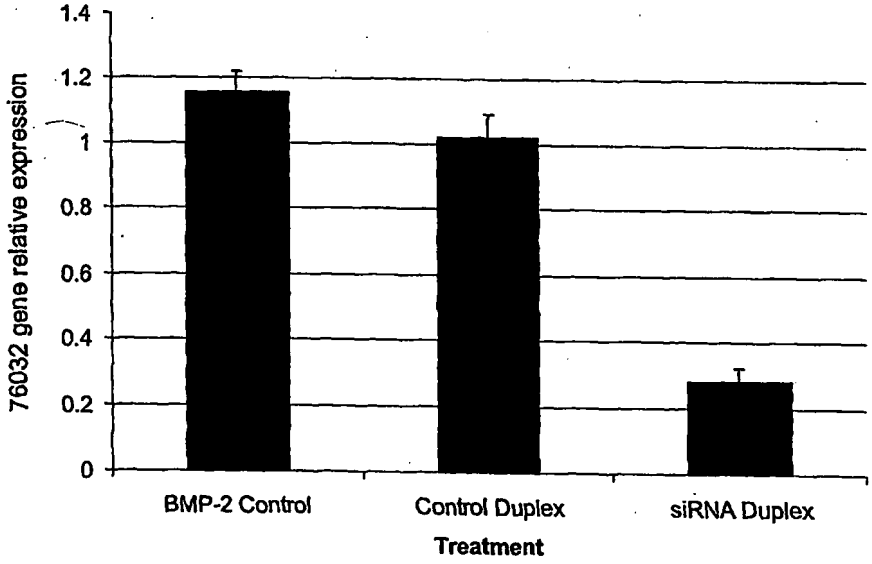
4
3
2
1
0
BMP-2 Control
Control Duplex
siRNA Duplex
Treatment

B


76032 gene relative expression
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1.2
1
0.8
0.6
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0
BMP-2 Control
Control Duplex
siRNA Duplex
Treatment

SEQUENCE LISTING

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Mertz, Lawrence
Jaiswal, Neelam
Houghton, Adam
Ji, Darren
Cook, Jonathan S.
Axelrod, Douglas W.

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Ser Glu Gly Gly Lys Leu Val Ile Lys Asp His Asp Glu Pro Ile Val
        80                  85                  90

ttg cga acc cgg cac atc ctg att gac aac gga gga gag ctg cat gct      577
Leu Arg Thr Arg His Ile Leu Ile Asp Asn Gly Gly Glu Leu His Ala
    95                  100                 105

ggg agt gcc ctc tgc cct ttc cag ggc aat ttc acc atc att ttg tat      625
Gly Ser Ala Leu Cys Pro Phe Gln Gly Asn Phe Thr Ile Ile Leu Tyr
110                 115                 120                 125

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Gly Arg Ala Asp Glu Gly Ile Gln Pro Asp Pro Tyr Tyr Gly Leu Lys
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tac att ggg gtt ggt aaa gga ggc gct ctt gag ttg cat gga cag aaa      721
Tyr Ile Gly Val Gly Lys Gly Gly Ala Leu Glu Leu His Gly Gln Lys
            145                 150                 155

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        160                 165                 170

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Ala Glu Gly Gly Tyr Phe Phe Glu Arg Ser Trp Gly His Arg Gly Val
    175                 180                 185

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Ile Val His Val Ile Asp Pro Lys Ser Gly Thr Val Ile His Ser Asp
190                 195                 200                 205

cgg ttt gac acc tat aga tcc aag aaa gag agt gaa cgt ctg gtc cag      913
Arg Phe Asp Thr Tyr Arg Ser Lys Lys Glu Ser Glu Arg Leu Val Gln
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tat ttg aac gcg gtg ccc gat ggc agg atc ctt tct gtt gca gtg aat      961
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            225                 230                 235

gat gaa ggt tct cga aat ctg gat gac atg gcc agg aag gcg atg acc     1009
Asp Glu Gly Ser Arg Asn Leu Asp Asp Met Ala Arg Lys Ala Met Thr
        240                 245                 250

aaa ttg gga agc aaa cac ttc ctg cac ctt gga ttt aga cac cct tgg     1057
Lys Leu Gly Ser Lys His Phe Leu His Leu Gly Phe Arg His Pro Trp
    255                 260                 265

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Ser Phe Leu Thr Val Lys Gly Asn Pro Ser Ser Ser Val Glu Asp His
270                 275                 280                 285

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Ile Glu Tyr His Gly His Arg Gly Ser Ala Ala Ala Arg Val Phe Lys
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        320                 325                 330

gta tct cag act aaa ggt ggg gag aaa att tca gac ctc tgg aaa gct     1297
Val Ser Gln Thr Lys Gly Gly Glu Lys Ile Ser Asp Leu Trp Lys Ala
    335                 340                 345

cac cca gga aaa ata tgc aat cgt ccc att gat ata cag gcc act aca     1345
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350                 355                 360                 365

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Arg Asn Thr Phe Asp His Cys Leu Gly Leu Leu Val Lys Ser Gly Thr
            625                 630                 635

ctc ctc ccc tcg gac cgt gac agc aag atg tgc aag atg atc aca gag     2209
Leu Leu Pro Ser Asp Arg Asp Ser Lys Met Cys Lys Met Ile Thr Glu
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gac tcc tac cca ggg tac atc ccc aag ccc agg caa gac tgc aat gct     2257
Asp Ser Tyr Pro Gly Tyr Ile Pro Lys Pro Arg Gln Asp Cys Asn Ala
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gtg tcc acc ttc tgg atg gcc aat ccc aac aac aac ctc atc aac tgt     2305
Val Ser Thr Phe Trp Met Ala Asn Pro Asn Asn Asn Leu Ile Asn Cys
670                 675                 680                 685

gcc gct tca gga tct gag gaa act gga ttt tgg ttt att ttt cac cac     2353
Ala Ala Ser Gly Ser Glu Glu Thr Gly Phe Trp Phe Ile Phe His His
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gta cca acg ggc ccc tcc gtg gga atg tac tcc cca ggt tat tca gag     2401
Val Pro Thr Gly Pro Ser Val Gly Met Tyr Ser Pro Gly Tyr Ser Glu
            705                 710                 715

cac att cca ctg gga aaa ttc tat aac aac cga gca cat tcc aac tac     2449
His Ile Pro Leu Gly Lys Phe Tyr Asn Asn Arg Ala His Ser Asn Tyr
        720                 725                 730

cgg gct ggc atg atc ata gac aac gga gtc aaa acc acc gag gcc tct     2497
Arg Ala Gly Met Ile Ile Asp Asn Gly Val Lys Thr Thr Glu Ala Ser
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gcc aag gac aag cgg ccg ttc ctc tca atc atc tct gcc aga tac agc     2545
Ala Lys Asp Lys Arg Pro Phe Leu Ser Ile Ile Ser Ala Arg Tyr Ser
750                 755                 760                 765

cct cac cag gac gcc gac ccg ctg aag ccc cgg gag ccg gcc atc atc     2593
Pro His Gln Asp Ala Asp Pro Leu Lys Pro Arg Glu Pro Ala Ile Ile
                770                 775                 780

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Arg His Phe Ile Ala Tyr Lys Asn Gln Asp His Gly Ala Trp Leu Arg
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Gly Gly Asp Val Trp Leu Asp Ser Cys Arg Phe Ala Asp Asn Gly Ile
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ggc ctg acc ctg gcc agt ggt gga acc ttc ccg tat gac gac ggc tcc     2737
Gly Leu Thr Leu Ala Ser Gly Gly Thr Phe Pro Tyr Asp Asp Gly Ser
    815                 820                 825

aag caa gag ata aag aac agc ttg ttt gtt ggc gag agt ggc aac gtg     2785
Lys Gln Glu Ile Lys Asn Ser Leu Phe Val Gly Glu Ser Gly Asn Val
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ggg acg gaa atg atg gac aat agg atc tgg ggc cct ggc ggc ttg gac     2833
Gly Thr Glu Met Met Asp Asn Arg Ile Trp Gly Pro Gly Gly Leu Asp
                850                 855                 860

cat agc gga agg acc ctc cct ata ggc cag aat ttt cca att aga gga     2881
His Ser Gly Arg Thr Leu Pro Ile Gly Gln Asn Phe Pro Ile Arg Gly
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att cag tta tat gat ggc ccc atc aac atc caa aac tgc act ttc cga     2929
Ile Gln Leu Tyr Asp Gly Pro Ile Asn Ile Gln Asn Cys Thr Phe Arg
        880                 885                 890

aag ttt gtg gcc ctg gag ggc cgg cac acc agc gcc ctg gcc ttc cgc     2977
Lys Phe Val Ala Leu Glu Gly Arg His Thr Ser Ala Leu Ala Phe Arg
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ctg aat aat gcc tgg cag agc tgc ccc cat aac aac gtg acc ggc att     3025
Leu Asn Asn Ala Trp Gln Ser Cys Pro His Asn Asn Val Thr Gly Ile
910                 915                 920                 925

gcc ttt gag gac gtt ccg att act tcc aga gtg ttc ttc gga gag cct     3073
Ala Phe Glu Asp Val Pro Ile Thr Ser Arg Val Phe Phe Gly Glu Pro
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ggg ccc tgg ttc aac cag ctg gac atg gat ggg gat aag aca tct gtg     3121
Gly Pro Trp Phe Asn Gln Leu Asp Met Asp Gly Asp Lys Thr Ser Val
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ttc cat gac gtc gac ggc tcc gtg tcc gag tac cct ggc tcc tac ctc     3169
Phe His Asp Val Asp Gly Ser Val Ser Glu Tyr Pro Gly Ser Tyr Leu
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acg aag aat gac aac tgg ctg gtc cgg cac cca gac tgc atc aat gtt     3217
Thr Lys Asn Asp Asn Trp Leu Val Arg His Pro Asp Cys Ile Asn Val
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ccc gac tgg aga ggg gcc att tgc agt ggg tgc  tat gca cag atg tac    3265
Pro Asp Trp Arg Gly Ala Ile Cys Ser Gly Cys  Tyr Ala Gln Met Tyr
990                 995                 1000                1005

att caa gcc tac aag  acc agt aac ctg cga  atg aag atc atc aag       3310
Ile Gln Ala Tyr Lys  Thr Ser Asn Leu Arg  Met Lys Ile Ile Lys
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aat gac ttc ccc agc  cac cct ctt tac ctg  gag ggg gcg ctc acc       3355
Asn Asp Phe Pro Ser  His Pro Leu Tyr Leu  Glu Gly Ala Leu Thr
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Arg Ser Thr His Tyr  Gln Gln Tyr Gln Pro  Val Val Thr Leu Gln
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aag ggc tac acc atc  cac tgg gac cag acg  gcc ccc gcc gaa ctc      3445
Lys Gly Tyr Thr Ile  His Trp Asp Gln Thr  Ala Pro Ala Glu Leu
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gcc atc tgg ctc atc  aac ttc aac aag ggc  gac tgg atc cga gtg      3490
Ala Ile Trp Leu Ile  Asn Phe Asn Lys Gly  Asp Trp Ile Arg Val
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Gly Leu Cys Tyr Pro  Arg Gly Thr Thr Phe  Ser Ile Leu Ser Asp
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Val His Asn Arg Leu  Leu Lys Gln Thr Ser  Lys Thr Gly Val Phe
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Val Arg Thr Leu Gln  Met Asp Lys Val Glu  Gln Ser Tyr Pro Gly
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Arg Ser His Tyr Tyr  Trp Asp Glu Asp Ser  Gly Leu Leu Phe Leu
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aag ctg aaa gct cag  aac gag aga gag aag  ttt gct ttc tgc tcc      3715
Lys Leu Lys Ala Gln  Asn Glu Arg Glu Lys  Phe Ala Phe Cys Ser
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atg aaa ggc tgt gag  agg ata aag att aaa  gct ctg att cca aag      3760
Met Lys Gly Cys Glu  Arg Ile Lys Ile Lys  Ala Leu Ile Pro Lys
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aac gca ggc gtc agt  gac tgc aca gcc aca  gct tac ccc aag ttc      3805
Asn Ala Gly Val Ser  Asp Cys Thr Ala Thr  Ala Tyr Pro Lys Phe
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acc gag agg gct gtc  gta gac gtg ccg atg  ccc aag aag ctc ttt      3850
Thr Glu Arg Ala Val  Val Asp Val Pro Met  Pro Lys Lys Leu Phe
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ggt tct cag ctg aaa  aca aag gac cat ttc  ttg gag gtg aag atg      3895
Gly Ser Gln Leu Lys  Thr Lys Asp His Phe  Leu Glu Val Lys Met
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gag agt tcc aag cag  cac ttc ttc cac ctc  tgg aac gac ttc gct      3940
Glu Ser Ser Lys Gln  His Phe Phe His Leu  Trp Asn Asp Phe Ala
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tac att gaa gtg gat  ggg aag aag tac ccc  agt tcg gag gat ggc      3985
Tyr Ile Glu Val Asp  Gly Lys Lys Tyr Pro  Ser Ser Glu Asp Gly
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atc cag gtg gtg gtg  att gac ggg aac caa  ggg cgc gtg gtg agc      4030
Ile Gln Val Val Val  Ile Asp Gly Asn Gln  Gly Arg Val Val Ser
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cac acg agc ttc agg  aac tcc att ctg caa  ggc ata cca tgg cag       4075
His Thr Ser Phe Arg  Asn Ser Ile Leu Gln  Gly Ile Pro Trp Gln
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ctt ttc aac tat gtg  gcg acc atc cct gac  aat tcc ata gtg ctt       4120
Leu Phe Asn Tyr Val  Ala Thr Ile Pro Asp  Asn Ser Ile Val Leu
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atg gca tca aag gga  aga tac gtc tcc aga  ggc cca tgg acc aga       4165
Met Ala Ser Lys Gly  Arg Tyr Val Ser Arg  Gly Pro Trp Thr Arg
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gtg ctg gaa aag ctt  ggg gca gac agg ggt  ctc aag ttg aaa gag       4210
Val Leu Glu Lys Leu  Gly Ala Asp Arg Gly  Leu Lys Leu Lys Glu
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caa atg gca ttc gtt  ggc ttc aaa ggc agc  ttc cgg ccc atc tgg       4255
Gln Met Ala Phe Val  Gly Phe Lys Gly Ser  Phe Arg Pro Ile Trp
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gtg aca ctg gac act  gag gat cac aaa gcc  aaa atc ttc caa gtt       4300
Val Thr Leu Asp Thr  Glu Asp His Lys Ala  Lys Ile Phe Gln Val
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Val Pro Ile Pro Val  Val Lys Lys Lys Lys  Leu
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Gly His Asp Gln Asp His His Val His Ile Gly Gln Gly Lys Thr Leu
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Leu Leu Thr Ser Ser Ala Thr Val Tyr Ser Ile His Ile Ser Glu Gly
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Gly Lys Leu Val Ile Lys Asp His Asp Glu Pro Ile Val Leu Arg Thr
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Arg His Ile Leu Ile Asp Asn Gly Gly Glu Leu His Ala Gly Ser Ala
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Asp Glu Gly Ile Gln Pro Asp Pro Tyr Tyr Gly Leu Lys Tyr Ile Gly
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Glu Ile Asp Gly Val Asp Met Arg Ala Glu Val Gly Leu Leu Ser Arg
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Asn His Ile Cys Asn Phe Phe Asp Phe Asp Thr Phe Gly Gly His Ile
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Ile Arg Asp Leu Ser Ile His His Thr Phe Ser Arg Cys Val Thr Val
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Phe Asp His Cys Leu Gly Leu Leu Val Lys Ser Gly Thr Leu Leu Pro
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Pro Gly Tyr Ile Pro Lys Pro Arg Gln Asp Cys Asn Ala Val Ser Thr
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Phe Trp Met Ala Asn Pro Asn Asn Asn Leu Ile Asn Cys Ala Ala Ser
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Gly Ser Glu Glu Thr Gly Phe Trp Phe Ile Phe His His Val Pro Thr
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Gly Pro Ser Val Gly Met Tyr Ser Pro Gly Tyr Ser Glu His Ile Pro

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Leu Gly Lys Phe Tyr Asn Asn Arg Ala His Ser Asn Tyr Arg Ala Gly
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Lys Arg Pro Phe Leu Ser Ile Ile Ser Ala Arg Tyr Ser Pro His Gln
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Asp Ala Asp Pro Leu Lys Pro Arg Glu Pro Ala Ile Ile Arg His Phe
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Tyr Asp Gly Pro Ile Asn Ile Gln Asn Cys Thr Phe Arg Lys Phe Val
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Ala Leu Glu Gly Arg His Thr Ser Ala Leu Ala Phe Arg Leu Asn Asn
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Phe Asn Gln Leu Asp Met Asp Gly Asp Lys Thr Ser Val Phe His Asp
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